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(57) Abstract

The present invention provides an isolated nucleic acid encoding a BCNG protein or a portion thereof or BCNG–related protein or a portion thereof. The present invention further provides a method for identifying a nucleic acid in a sample which encodes a BCNG protein or a BCNG–related protein. The present invention also provides a method for testing whether a compound affects the expression of a BCNG protein or a BCNG–related protein. In addition, the present invention further provides a method for identifying a compound capable of interacting with a BCNG protein or a BCNG–related protein. Also, the present invention provides a method for identifying a compound capable of modulating BCNG protein or BCNG–related protein activity. Further, the present invention also provides a method of treating a condition in a subject which comprises administering to the subject an amount of the provided compound, effective to treat the condition. Finally, the present invention provides a pharmaceutical composition which comprises the provided compound and a pharmaceutically acceptable carrier.

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BRAIN OR HEART CYCLIC NUCLEOTIDE GATED ION CHANNEL COMPOUNDS AND USES THEREOF

This application claims priority of U.S. Serial No. 09/086,436, filed May 28, 1998, which is a continuation-in-part of U.S. Serial No. 08/997,685, filed December 23, 1997, the content of which is hereby incorporated into this application by reference.

Throughout this application, various publications are referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding the Sequence Listing and the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art.

Background of the Invention

Introduction

Ion channels are a diverse group of proteins that regulate the flow of ions across cellular membranes. In the nervous system, ion channel activity has evolved into a rapid and accurate system for intercellular communication. The electrical excitability characteristics of each neuron is in part determined by the set of channels it expresses. However, cells are also able to regulate the activity of individual channels in response to physiological or developmental events, and there is growing evidence that ion channels can be the site of integration of multiple electrical and biochemical pathways.

In vivo, ion channels appear to be multimeric proteins that are comprised of several distinct gene families, coding for channels with distinct structural and functional properties. Within a gene family, the potential for heterogeneity arising from the

combinatorial assembly of different pore-forming and auxiliary subunits (Greene, et al., 1995). Channel properties can be modulated by second messenger cascades and can directly bind intracellular proteins such as kinases suggesting that this may be an important way to efficiently target the signaling cascade to its effector molecule. The electrical characteristics of each neuron is, in part, determined by the set of ion channels that it expresses. However, cells are also able to regulate the activity of individual channels in response to physiological or developmental events; pore-forming (α) subunits can interact with a variety of intracellular proteins, including auxiliary (β) subunits, cytoskeleton-associated proteins and protein kinases (Greene, et al., 1995). In addition to auxiliary (β) subunits, pore-forming subunits can interact with a variety of intracellular proteins and second messenger molecules themselves including G-proteins, cytoskeleton-associated proteins and protein-kinases (Adelman, et al., 1995).

Several classes of ion channels bind directly, and are regulated by, second messenger molecules such as cyclic nucleotides (Zagotta, et al., 1996; Bruggemann, et al., 1993, and Hoshi, et al., 1995) or Ca^{+2} (Adelman, et al., 1992; Kohler, et al., 1996). Channels with this property may be key elements in the control of neuronal signaling, as they directly couple biochemical cascades with electrical activity. Cyclic nucleotide-gated channels (CNG) play a distinct role both in visual and olfactory signal transduction; their recent identification in the hippocampus and other regions of the brain, where cAMP and cGMP are known to mediate different forms of synaptic plasticity (Krapivinisky, et al., 1995; Frey, et al., 1993; Bolshakov, et al., 1997; and Arancio, et al., 1995), suggests that CNG-channels

may also contribute to the regulation of excitability in central neurons (Kingston, et al., 1996 and Bradley, et al., 1997).

The first structural gene for a K^+ channel to be isolated was the gene encoded by the Shaker (Sh) locus in Drosophila melanogaster (Strong, et al., 1993; Papazian, et al., 1987). Its sequence is the prototype of a large and still expanding family of related genes (Kamb, et al., 1987; Warmke, et al., 1994). The properties of a number of well characterized K^+ currents, that still await a molecular definition, predicts that other members of this family are yet to be identified (Atkinson, et al., 1991).

Although the initial members of the K^+ channel superfamily were cloned by chromosomal localization of alleles responsible for functional defects (Sh, eag and slo from Drosophila; (Papazian, et al., 1987; Kamb, et al., 1987; Warmke, et al., 1991; Atkinson, et al., 1991) or following the purification of a relatively abundant protein such as the cGMP-channel from bovine retina (Liman, et al., 1994), the most widely used strategy for cloning new members of the K^+ channel superfamily is by homology to these sequences. Unfortunately, this approach is not well suited for identifying more divergent sequences and potentially new branches in the phylogenetic tree of the K^+ channel superfamily. Expression cloning in *Xenopus* oocytes can circumvent this problem; this implies a pre-existing or readily detectable physiological characterization of the channel.

Summary of the Invention

The present invention provides an isolated nucleic acid encoding a BCNG protein or a portion thereof. The present invention further provides an isolated nucleic acid encoding a BCNG-related protein or a portion thereof. Further, the present invention provides a vector, which comprises cDNA encoding mBCNG-1 (ATCC Accession No. 209781). In addition, the present invention further provides a vector, which comprises cDNA encoding hBCNG-1 (ATCC Accession No. 209827). The present invention also provides an isolated BCNG protein. Further, the present invention also provides an isolated BCNG-related protein.

The present invention additionally provides a composition comprising a nucleic acid encoding a BCNG protein or a portion thereof, or a BCNG-related protein or a portion thereof and a carrier. In addition, the present invention further provides a composition comprising a BCNG protein or a portion thereof, or a BCNG-related protein or portion thereof and a carrier.

Additionally, the present invention provides a nucleic acid probe capable of specifically hybridizing with a nucleic acid encoding a BCNG protein or BCNG-related protein.

The present invention provides a method for identifying a nucleic acid in a sample which encodes a BCNG protein or a BCNG-related protein which comprises: (a) contacting the sample with a nucleic acid probe capable of specifically hybridizing with nucleic acid encoding a BCNG protein or a BCNG-related protein under conditions permissive to the formation of a complex between the nucleic acid probe and the nucleic acid encoding the BCNG

protein or the BCNG-related protein in the sample; (b) determining the amount of complex formed in step (a); and (c) comparing the amount of complex determined in step(b) with the amount of complex formed using an arbitrary sequence, a greater amount of complex formed with the BCNG-specific probe indicating the presence of a nucleic acid encoding a BCNG protein or a BCNG-related protein in the sample.

Further, the present invention provides a method for testing whether a compound affects the expression of a BCNG protein or a BCNG-related protein which comprises: (a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound; (b) determining the amount of expression of BCNG protein or BCNG-related protein in the sample; and (c) comparing the amount of BCNG protein or BCNG-related protein expression determined in step (b) with the amount determined in the absence of the compound.

In addition, the present invention further provides a method for identifying a compound capable of interacting with a BCNG protein or a BCNG-related protein which comprises: (a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound under conditions permissive to formation of a complex between the compound and the BCNG protein or the BCNG-related protein; (b) determining the amount of complex formed between the compound and the BCNG protein or the BCNG-related protein; (c) comparing the amount of complex formed in step (b) with the amount formed in the absence of the compound, a greater amount of complex formed in the presence of the compound indicating the presence of a compound capable of interacting with a BCNG protein or a BCNG-related protein.

Also, the present invention provides a method for identifying a compound capable of modulating BCNG protein or BCNG-related protein activity which comprises: (a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound; (b) determining the amount of activity of the BCNG protein or BCNG-related protein in the sample; and (c) comparing the amount of activity of the BCNG protein or the BCNG-related protein determined in step (b) with the amount determined in the absence of the compound, an increase or decrease in activity indicating the presence of a compound capable of modulating the activity of the BCNG protein or the BCNG-related protein.

Further, the present invention also provides a method of treating a condition in a subject which comprises administering to the subject an amount of the provided compound, effective to treat the condition.

Finally, the present invention provides a pharmaceutical composition which comprises the provided compound and a pharmaceutically acceptable carrier.

Brief Description of the Figures

Figures 1A-1D. Primary structure of mBCNG-1. **Figure 1A**. Deduced amino acid sequence (Seq.ID.No.:2) encoded by the mBCNG-1 cDNA. The seven hydrophobic domains, homologous to the six transmembrane domains (S1-S6) and the pore (P) of K^+ channels, are indicated (___). The putative cyclic-nucleotide binding site (CNBs) is marked by an (- - -), C-terminal prolines (. . .) the consensus N-glycosylation site with presumptive extracellular localization (*) are also marked. **Figure 1B**. Kyte and Doolittle hydropathy plot of the predicted amino acid sequence of mBCNG-1. The profile was generated by the Kyte and Doolittle method with a window size of 7 amino acids. The numbers on the top line indicate the position in the mBCNG-1 sequence. Hydrophobic regions corresponding to S1 through S6 and the P region lie below the zero line while the N-glycosylation site (*) is in a hydrophilic region between S5 and P. Numbering (top line) indicate position in the mBCNG-1 sequence. Profile generated with a window size of 7 residues. **Figure 1C**. Multiple alignment of the putative P region of mBCNG-1 with the P regions of *Drosophila* Eag (DEAG), mouse Eag (MEAG), human Erg (HERG), α-subunit of bovine retinal CNG-channel (BRET-1), and β-subunit of human retinal CNG-channel (HRET-2). Arrowheads mark the residues 344 and 352 (see Example 1). **Figure 1D**. Alignment of the (CNBs) of BCNG-1 with the corresponding site in the rat olfactory CNG-channel (ROLF-1), bovine cGMP-dependent protein kinase (PKG), bovine cAMP-dependent protein kinase (PKA), and catabolite activator protein of E.coli (CAP). Continuous lines mark α-helical (α) and β-strand (β) elements of the secondary structure elements of CAP, while asterisks indicate specific amino acids that appear to lie close to the cAMP molecule in the CAP crystal structure.

Figures 2A-2D. mBCNG-1 is a 132 kDa glycosylated protein. **Figure 2A**. Western blot analysis of BCNG-1 protein in a mouse brain extract. Ten μg of a total brain SDS-extract was loaded per strip then probed with αq1 (1) or αq2 (3) antiserum or, strip 2 with αq1 (2) or αq2 (4) antiserum preadsorbed with the GST-d5 fusion protein. The arrow marks the position of the specific signal, of corresponding to the native mBCNG-1 protein. **Figure 2B**. Western blot using the αq1 antiserum against: total brain extract (1), total brain extract pre-treated with N-glycosidase F (2) and *in vitro* translated mBCNG-1 protein (3). Positions of molecular weight standards are shown on the left. Also shown, Western blot containing 10 μg of proteins from each of the indicated brain tissues which was tested with antisera against mBCNG-1 and showing widespread expression of the mBCNG-1 protein in mouse brain. **Figure 2C**. indicates reactivity with αq1. **Figure 2D**. indicates reactivity with αq2.

Figure 3. Northern blot analysis of mBCNG-1 expression in
different mouse tissues. Two μg of poly(A)$^+$ RNA from each of each of the following tissues was used: heart (H), brain (B), spleen (S), lung (Lu), liver (Li), skeletal muscle (M), kidney (K) and testis (T) were loaded. The filter was probed with a DNA fragment encoding amino acids 6-131 of the mBCNG-1 sequence. A probe corresponding to amino acids 594- 720 recognized the same bands, confirming that the cDNA fragments isolated from the λgt10 and pJG4-5 libraries are from a contiguous mRNA sequence. Positions of molecular weight standards are shown on the left.

Figure 4. *In situ* hybridization analysis of mBCNG-1 expression in the brain. Parasagittal section of a mouse brain probed with an antisense oligonucleotide directed to the mRNA region corresponding to amino acids 648-657

of the mBCNG-1 sequence. Abbreviations: nCtx, neocortex; Hp, hippocampus; Crb, cerebellum; BrSt, brainstem.

Figures 5A-5F: Immunohistochemical analysis of mBCNG-1 expression in the brain. Parasagittal sections of a mouse brain were stained with αq1 and αq2 antisera. The patterns of mBCNG-1 expression detected with the two different antisera were identical, and in both cases the staining was entirely abolished by preadsorbing the sera with the GST-d5 fusion protein. mBCNG-1 immunoreactivity in the cerebral cortex. **Figure 5C-5D**. mBCNG-1 immunoreactivity in the hippocampus. In Figure 5C, the arrow shows the position of the hippocampal fissure; areas CA_1, CA_3 and dentate gyrus (DG) are labeled. Figure 5D shows a detail of the *stratum pyramidale* of area CA_3. **Figure 5E-5F**. mBCNG-1 immunoreactivity in the cerebellum. (Figure 5A, Figure 5C, Figure 5E: 60X; Figure 5B, Figure 5D, Figure 5F: 100X) magnification).

Figures 6A-6B. Southern blot analysis of mouse genomic DNA.
4μg of mouse genomic DNA were loaded onto each lane following digested with Eco RI (1), Hind III (2), Bam HI (3), Pst I (4) or Bgl II (5). The filter was probed with a DNA fragment encoding amino acids 269-462 of the BCNG-1 sequence at high (Figure 6A) and (Figure 6B) low stringency. Positions of molecular weight standards are shown on the left.

FIGURES 7A-7C. Schematic representation of the mouse and human BCNG clones. **Figures 7A-7B**. Predicted structure of mBCNG-1 (Santoro et al., 1997) with six transmembrane domains (S1-S6), pore region (P), cyclic nucleotide binding site (CNBs) and long C-terminal tail, including a polyglutamine stretch (Q). The predicted sequences encoded by the partial cDNA clones of three other mouse and two human BCNG genes are shown in a tentative

alignment to mBCNG-1. Lines with double-headed arrows above the sequences indicate if the fragment was obtained from a cDNA library (λgt10 or pJG4-5), RT-PCR reaction, or EST database. Dashed lines with double-headed arrows underneath the sequences indicate the position of probes used herein (see Examples 1-5 in Experimental Details section). Hashed box in the 5' region of mBCNG-4 indicates the position of the probable intron in the M28-EST clone. **Figure 7C**. Percent sequence similarity among the mouse and human BCNG genes. The alignments were performed by comparing only the core region of the proteins, corresponding to amino acids 111-419 (numbering according to mBCNG-1, see Figure 8), and including transmembrane domains S1-S6. The mBCNG-4 sequence was not included in this alignment. However, limited alignment within the available cyclic nucleotide binding domain sequence of mBCNG-4 (aa 529-592, numbering according to mBCNG-1, see Figure 8) shows a 79% similarity to mBCNG-1.

FIGURES 8A-8B. Mouse and human BCNG protein alignments. Tentative alignment of the predicted amino acid sequences for the four mouse (mBCNG-1, 2, 3 and 4) and two human genes (hBCNG-1 and 2). The proposed structural features of the protein (putative transmembrane regions, pore region and cyclic nucleotide binding site) are indicated (see also Fig. 5). (-) indicates residues identical to mBCNG-1; divergent residues are otherwise reported. (.) indicates a gap (or deletion) in the aligned sequences. (*) at end of sequence indicates stop codon. (*) above position 327 marks N-glycosylation site of mBCNG-1. The arrow marks the single consensus PKA phosphorylation site present in BCNG-1 and BCNG-2.

FIGURES 9A-9D. Northern Blot Analysis of Mouse BCNG Gene Expression. Multiple Tissue Northern blot, containing 2 µg of polyA+ RNA from each of the following mouse

tissues: heart (He), brain (Br), spleen (Sp), lung (Lu) liver (Li), skeletal muscle (Mu), kidney (Ki) and testis (Te), was hybridized to DNA fragments corresponding to the indicated BCNG genes. Molecular size markers are indicated on the left.

FIGURES 10A-D. Northern Blot Analysis of Human BCNG Gene Expression. **Figures 10A-10B**. Multiple human tissue Northern blot, containing 2 mg of polyA+ RNA from each of the following tissues: heart (He), brain (Br), placenta (Pl), lung (Lu) liver (Li), skeletal muscle (Mu), kidney (Ki) and pancreas (Pa), was hybridized to DNA fragments corresponding to the indicated BCNG genes. **Figures 10C-10D**. The same fragments were used to probe a human Brain Multiple Tissue blot, containing 2 µg of polyA+ RNA from each of the following tissues: amygdala (Am), caudate nucleus (Cn), corpus callosum (CC), hippocampus (Hi), total brain (Br), substantia nigra (SN), subthalamic nucleus (Sn) and thalamus (Th). Molecular size markers are indicated on the left.

FIGURE 11. Schematic representation of the general architecture of the BCNG channel proteins based on homology to the voltage-gated K^+channels and the cyclic nucleotide-gated channels.

FIGURE 12. Alignment of the S4 voltage sensing regions of the prototypical voltage- gated K^+channel shaker and cyclic nucleotide-gated channel bRET1 with the S4 sequence of mBCNG-1 (Seq.ID.No.:2). Boxed residues are positively charged amino acids present in one or more of the S4 sequences. The stars indicate the position of amino acids with negatively charged acidic side chains that are present in the bRET1 sequence.

FIGURES 13A-13B. **Figure 13A.** Sequence alignment of functional cyclic nucleotide binding sites from catabolite activating protein (CAP, Aiba et al., 1982; Cossart & Gicquel, 1982), A and B sites of recombinant bovine R1α (PKAa and PKAb, Titani et al., 1984), bovine retinal channel α subunit (bRET1, Kaupp et al.,1989) and the catfish olfactory α subunit (fOLF1, Goulding et al., 1992) along with the putative cyclic nucleotide binding sites of drosophila Ether-a-gogo (dEAG, Warmke et al., 1991), Arabidopsis Thaliana K transport protein (KAT1, Anderson et al., 1992) and mBCNG-1 (Seq.ID.No.:2)(described herein). The six residues that are totally conserved across all of the binding sites whose functional competence has been unequivocally confirmed are marked by asterisks. The conserved arginine that forms an ionic bond with the cyclic nucleotide is indicated by an arrow labeled R559. The residue in the third (C) α-helix that has been shown to influence coupling of activation to cAMP versus cGMP binding is indicated by an arrow labeled D604 (the cGMP selective substitution in bRET1). **Figure 13B.** Schematic representation of the cyclic nucleotide binding site of bRET1 showing the critical interactions between the binding site and the cyclic nucleotide. This model of the binding pocket is based on the crystal structure of CAP and bovine R1α. The cGMP is shown bound in an extended - or anti - form with the cyclized phosphate making an ionic bond with Arginine559 (bRET1 numbering) and the purine ring forming favorable contacts with D604 in this cGMP selective channel.

FIGURE 14. Alignment of the P loop pore forming regions of the prototypical voltage-gated K^+channel shaker and cyclic nucleotide-gated channel bRET1 with the S4

sequence of mBCNG1. The aligned channels are mBCNG-1 (Seq.ID.No.:2), Shaker (SHAK, Papazian et al., 1987; Kamb et al., 1988), SHAW, Wei, et al., 1990) calcium activated K channel (MSLO, Pallanck and Ganetzky, 1994) the plant inward rectifier (AKT Sentenac et al., 1992), drosophila and mouse ether-a-gogo's (DEAG, Warmke et al., 1991; MEAG, Warmke and Ganetzky, 1994) Human ether-a-gogo related gene (HERG, Warmke and Ganetzky, 1994), bovine retinal α-subunit (bRET1, Kaupp et al., 1989) and human retinal β subunit (HRET-2, Chen et al., 1993). The arrows mark positions where the BCNG channels show pronounced and potentially important changes in sequence from the normal motif seen in K^+ selective channels.

FIGURES 15A-15B. Schematic representation of repetitive firing of a pacemaker neuron and its involvement in the generation and regulation of rhythmic firing patterns. **Figure 15A**. Shows that Ih activation upon hyperpolarization following an action potential. As this is a non-selective cationic current, it carries an inward current at these potentials which leads to the depolarization of the cell back towards the threshold for firing of the next action potential. **Figure 15B**. Shows the action of sympathetic and vagal stimulation on the activity of cardiocytes from the sinoatrial node - the pacemaker area of the heart. Norepinephrine (NE) leads to a shift in the activation of Ih towards more depolarized potentials which accelerates the return to the action potential firing threshold, and hence, leads to an acceleration of the firing rate. In contrast, acetylcholine (ACh) shifts the activation of Ih to more hyperpolarized potentials. Thus, the current will turn on later during the repolarization phase delaying the return to threshold - the firing rate of the cell will thus be slowed. These changes in the activation

properties of Ih are thought to be due to changes in the concentration of cAMP with ACh lowering the concentration and NE increasing the concentration which has been shown to alter the activation properties of Ih. ("Principles of Neural Science" by Kandel, Schwartz and Jessell 1991).

FIGURES 16A-16F. mBCNG-1 expression gives rise to a hyperpolarization-activated current that resembles the native neuronal pacemaker current. **Figure 16A**. Currents elicited by 3 s hyperpolarizations from a holding potential of -40 mV to potentials ranging from -60 to -130 mV in 5 mV increments. **Figure 16B**. Relation between steady-state current at end of hyperpolarizing step and patch voltage. **Figure 16C**. Tail currents recorded upon return to -40 mV following hyperpolarizations to various test voltages. Records shown on an expanded time scale to emphasize tail currents. **Figure 16D**. Mean relation between tail current amplitude and voltage during hyperpolarizing step. For each patch, tail current data were normalized to the maximal tail current amplitude obtained from a fit of the Boltzmann equation (see Example 4, Experimental Details section). Normalized tail currents were then averaged for 5 patches. Mean V1/2 = -100.0 mV, slope = 5.8 mV. **Figure 16E**. Activation time course of mBCNG-1 currents. Data were sampled at 5 kHz and the currents during voltage steps between -105 to -130mV were fitted by single exponential functions (smooth lines), after allowing for an initial lag. **Figure 16F**. Relationship between mean time constants of activation and voltage. In the above experiments, the extracellular solution was KCl/NaCl-CaCl2, and the intracellular solution was KCl/NaCl-EGTA (See, Example 4, Experimental Procedures).

FIGURES 17A-17C. mBCNG-1 is a cation channel that selects

weakly for potassium over sodium. **Figure 17A**. Tail currents obtained upon depolarizing steps to various test potentials following a 0.3 sec step to -130 mV to activate the mBCNG-1 current. Test potentials ranged from -60 to + 20 mV in 5 mV increments (indicated next to alternate current traces). The extracellular solution was NaCl-EGTA while the intracellular solution was KCl-EGTA. **Figure 17B**. Similar tail current protocol used to measure reversal potential after switching to the low Cl, KAspartate-EGTA solution in the bath. **Figure 17C**. Tail current amplitude as a function of membrane voltage during tail. Open symbols represent the current amplitudes determined with the KCl-EGTA solution in the bath (o, initial measurement, E_{rev} = -32.8 ± 2.5, n = 3; □, following washout of the KAspartate-EGTA solution, E_{rev} = -31.2 ± 1.6, n = 3). The filled circles represent the measurements made in the presence of the K-Aspartate solution (E_{rev} = -28.2 ± 1.6, n = 4). In all three panels, the zero current level is indicated by a horizontal dashed line.

FIGURES 18A-18C. The mBCNG-1 channel is blocked by external Cs but not by external Ba, similar to native pacemaker channels. mBCNG-1 current records from an outside-out patch. In Figures 18A-18C, the current at the holding potential (-40 mV) and in response to a hyperpolarization to -130 mV are superimposed. **Figure 18A and 18B**. mBCNG-1 current records from an outside-out patch. Currents at the holding potential (-40 mV) and in response to a step to -130 mV are superimposed. The sequential records were obtained when the extracellular surface was exposed to the KCl/NaCl-CaCl2 solution (control), or solutions in which 2 mM CsCl iso-osmotically replaced NaCl or 1 mM BaCl2 replaced the CaCl2. The intracellular solution was KCl/NaCl-EGTA. **Figure 18C**. Dose response relationship for the

inhibition of mBCNG-1 current by Cs. Data are the mean from 6 patches (not all determined at each concentration). The solid line shows a fit of the Hill equation, $I/Imax = 1/\{1+(IC_{50}/[Cs])^n\}$, where [Cs] is the Cs concentration, IC_{50} is the half maximal inhibitory concentration of Cs, n is the Hill coefficient, I is the current in the presence of Cs and Imax is the uninhibited current. The fit yields an IC_{50} of 201 μM and a Hill coefficient of 1.08.

FIGURES 19A-19C. The mBCNG-1 channel is directly regulated by cytoplasmic cAMP. **FIGURE 19A**. mBCNG-1 current record from an inside-out patch in response to hyperpolarization to -100 mV in the absence or presence of 1 μM cAMP in the intracellular solution (KCl-EGTA). The extracellular solution was KCl-CaCl2. **FIGURE 19B**. Records from another patch in the absence and presence of 30 μM cAMP (same solutions as in A). **FIGURE 19C**. (Left panel) Tail current activation curves in the absence and presence of 1 μM cAMP for the patch shown in panel A. Data were analyzed and plotted as described in Fig. 1D. Curves fit by Boltzmann relation with following parameters: 0 μM cAMP: $V_{1/2}$ = -100 mV, slope = 5.4 mV and $I_{tail,\ max}$ = -33.8 pA; 1 μM cAMP: $V_{1/2}$ = -98 mV, slope = 5.3 and $I_{tail,\ max}$ = -33.7 pA. (Right panel) Mean tail current activation curves for patches in absence (solid circles) and presence (open circles) of cAMP. Data averaged from 5 patches. cAMP concentrations range from 1 - 3000 μM, for cAMP; $V_{1/2}$ = -98.3 mV, slope = 6 mV.

FIGURES 20 A-C. mBCNG-2 is expressed in the sinoatrial node of the heart. **FIGURE 20A.** PolyA+ RNA samples from ventricle, atrial and sino-atrial node of the rabbit heart were tested by RT-PCR for the presence of mBCNG-2 or 3 transcripts. Lanes: 1) molecular weight marker; 2) reaction performed in the absence of reverse transcriptase; 3) ventricle RNA; 4) atrial RNA; 5) sino-

atrial node RNA; 6) PCR reaction on plasmid containing mBCNG-1 cDNA; 7) PCR reaction on plasmid containing mBCNG-2 cDNA; 8) PCR reaction on plasmid containing mBCNG-3 cDNA. Molecular size markers are indicated on the left. The arrow on the right indicates the expected 340 bp amplification product. **FIGURE 20B.** Southern blot analysis of the gel shown in A using a probe to mBCNG-2. **FIGURE 20C.** Southern blot analysis of the gel in A using a probe to mBCNG-3.

Detailed Description of the Invention

The present invention provides an isolated nucleic acid encoding a BCNG protein or a portion thereof. The present invention further provides an isolated nucleic acid encoding a BCNG-related protein or a portion thereof.

In an embodiment of this invention, the BCNG protein is encoded by the sequence shown in mBCNG-1 (ATCC Accession No. 209781) (Seq.ID.No.:1), mBCNG-2 (Seq.ID.No.:5), mBCNG-3 (Seq.ID.No.:9), mBCNG-4 (Seq.ID.No.:11), hBCNG-1 (ATCC Accession No. 209827) (Seq.ID.No.:3) or hBCNG-2 (Seq.ID.No.:7). According to an embodiment of this invention, the nucleic acid is DNA or RNA. In an embodiment of the present invention, the nucleic acid is cDNA. According to an embodiment of this invention, the cDNA has the nucleotide sequence shown in SEQ. ID. No.: 1 for mBCNG-1 (ATCC Accession No. 209781) , SEQ. ID. No.: 3 for hBCNG-1, SEQ. ID. No.: 5 for mBCNG-2, SEQ. ID. No.: 7 for hBCNG-2, SEQ. ID. No.: 9 for mBCNG-3, or SEQ. ID. No.:11 for mBCNG-4. An embodiment of the present invention is a vector comprising the nucleic acid. According to an embodiment of this invention, the vector comprises viral or plasmid DNA. An embodiment of this invention comprises the nucleic acid and regulatory elements. One embodiment of this invention is a host vector system which comprises the provided expression vector in a suitable host.

Further, the present invention provides a vector, which comprises cDNA encoding mBCNG-1 (ATCC Accession No. 209781). In addition, the present invention further provides a vector, which comprises cDNA encoding hBCNG-1 (ATCC Accession No. 209827).

In an embodiment of this invention, the suitable host is a bacterial cell, a eukaryotic cell, a mammalian cell or an insect cell.

The present invention also provides an isolated BCNG protein. Further, the present invention also provides an isolated BCNG-related protein.

In one embodiment of this invention, the BCNG protein has the amino acid sequence shown in Seq.ID.No.:2 for mBCNG-1 (Figures 8A-8B), Seq.ID.No.:6 for mBCNG-2 (Figures 8A-8B), Seq.ID.No.:10 for mBCNG-3 (Figures 8A-8B), Seq.ID.No.12 for mBCNG-4 (Figures 8A-8B), Seq.ID.No.:4 for hBCNG-1 (Figures 8A-8B), or Seq.ID.No.:8 for hBCNG-2 (Figures 8A-8B). According to another embodiment of this invention, the BCNG-related protein has an amino acid sequence with substantial homology to the amino acid sequence shown in Seq.ID.No.:2 mBCNG-1 (Figures 8A-8B), Seq.ID.No.:6 for mBCNG-2 (Figures 8A-8B), Seq.ID.No.:10 for mBCNG-3 (Figures 8A-8B), Seq.ID.No.12 for mBCNG-4 (Figures 8A-8B), Seq.ID.No.:4 for hBCNG-1 (Figures 8A-8B), or Seq.ID.No.:8 for hBCNG-2 (Figures 8A-8B).

The present invention additionally provides a composition comprising a nucleic acid, encoding a BCNG protein or a portion thereof or a BCNG-related protein or a portion thereof and a carrier. In an embodiment of the present invention the nucleic acid comprises substantially the same coding sequence as the coding sequence shown in SEQ. ID. No.: 1 for mBCNG-1, SEQ. ID. No.: 3 for hBCNG-1, SEQ. ID. No.: 5 for mBCNG-2, SEQ. ID. No.: 7 for hBCNG-2, SEQ. ID. No.: 9 for mBCNG-3, SEQ. ID. No.:11 for mBCNG-4 or a portion of such coding sequence.

In addition, the present invention further provides a

composition comprising a BCNG protein or portion thereof or a BCNG-related protein or portion thereof and a carrier.

In an embodiment of this invention the BCNG protein comprises the amino acid sequence shown in Seq.ID.No.:2 mBCNG-1 (Figures 8A-8B), Seq.ID.No.:6 for mBCNG-2 (Figures 8A-8B), Seq.ID.No.:10 for mBCNG-3 (Figures 8A-8B), Seq.ID.No.12 for mBCNG-4 (Figures 8A-8B), Seq.ID.No.:4 for hBCNG-1 (Figures 8A-8B), Seq.ID.No.:8 for hBCNG-2 (Figures 8A-8B).

Additionally, the present invention provides a nucleic acid probe capable of specifically hybridizing with a nucleic acid encoding a BCNG protein or BCNG-related protein. One embodiment of this invention is a nucleic acid probe capable of specifically hybridizing with the provided nucleic acid. According to an embodiment of this invention the probe is capable of specifically hybridizing with the nucleic acid sequence shown in Seq.ID.No:13, Seq.ID.No:14, Seq.ID.No:15, Seq.ID.No:16, Seq.ID.No:17, Seq.ID.No:18, Seq.ID.No:19, Seq.ID.No:20, Seq.ID.No:21, Seq.ID.No:21, Seq.ID.No:22, Seq.ID.No:23, Seq.ID.No:24, Seq.ID.No:25, Seq.ID.No:26, Seq.ID.No:27, Seq.ID.No:28, Seq.ID.No:29, Seq.ID.No:30, Seq.ID.No:31, Seq.ID.No:32, Seq.ID.No:33, or Seq.ID.No:34.

The present invention provides a method for identifying a nucleic acid in a sample which encodes a BCNG protein or a BCNG-related protein which comprises: (a) contacting the sample with a nucleic acid probe capable of specifically hybridizing with nucleic acid encoding a BCNG protein or a BCNG-related protein under conditions permissive to the formation of a complex between the nucleic acid probe and the nucleic acid encoding the BCNG

protein or the BCNG-related protein in the sample; (b) determining the amount of complex formed in step (a); and (c) comparing the amount of complex determined in step(b) with the amount of complex formed using an arbitrary sequence, a greater amount of complex formed with the BCNG-specific probe indicating the presence of a nucleic acid encoding a BCNG protein or a BCNG-related protein in the sample.

In one embodiment of this invention, step (a) further comprises amplifying the nucleic acid molecule encoding the BCNG protein or the BCNG-related protein. According to an embodiment of this invention, the amplification comprises contacting the nucleic acid molecule from the sample with at least one amplification primer capable of specifically hybridizing to mBCNG-1 (Seq.ID.No.:1), mBCNG-2 (Seq.ID.No.:5), mBCNG-3 (Seq.ID.No.:9), mBCNG-4 (Seq.ID.No.:11), hBCNG-1 (Seq.ID.No.:3) or hBCNG-2 (Seq.ID.No.:7) under conditions suitable for polymerase chain reaction. In an embodiment of this invention, the amplified nucleic acid molecule encoding the BCNG protein or the BCNG-related protein is detected by size fractionation. One embodiment of this invention further comprises isolating the complex by size fractionation. According to an embodiment of this invention, the nucleic acid probe is labeled with a detectable marker. In an embodiment of this invention, the detectable marker is a radiolabeled molecule, a fluorescent molecule, an enzyme, a ligand, or a magnetic bead. According to an embodiment of this invention, the probe comprises the nucleotide sequence shown in Seq.ID.No:13, Seq.ID.No:14, Seq.ID.No:15, Seq.ID.No:16, Seq.ID.No:17, Seq.ID.No:18, Seq.ID.No:19, Seq.ID.No:20, Seq.ID.No:21, Seq.ID.No:21, Seq.ID.No:22, Seq.ID.No:23, Seq.ID.No:24, Seq.ID.No:25, Seq.ID.No:26, Seq.ID.No:27, Seq.ID.No:28, Seq.ID.No:29,

Seq.ID.No:30, Seq.ID.No:31, Seq.ID.No:32, Seq.ID.No:33, or Seq.ID.No:34. In an embodiment of the present invention, the nucleic acid probe is capable of specifically hybridizing to nucleic acid encoding mBCNG-1 (Seq.ID.No.:1), mBCNG-2 (Seq.ID.No.:5), mBCNG-3 (Seq.ID.No.:9), mBCNG-4 (Seq.ID.No.:11), hBCNG-1 (Seq.ID.No.:3) or hBCNG-2 (Seq.ID.No.:7). The present invention also provides an isolated nucleic acid, previously unknown, identified by the provided amplification method. In one embodiment, the sample comprises cells or cell extract or cell lysate or a tissue sample or a biological fluid sample.

Further, the present invention provides a method for testing whether a compound modulates the expression of a BCNG protein or a BCNG-related protein which comprises: (a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound; (b) determining the amount of expression of BCNG protein or BCNG-related protein in the sample; and (c) comparing the amount of BCNG protein or BCNG-related protein expression determined in step (b) with the amount determined in the absence of the compound thereby determining whether the compound modulates BCNG protein expression or BCNG-related protein expression. The present invention provides such a method for screening a large number of compounds to determine whether one or more of the compounds modulates the activity of a BCNG protein or a BCNG-related protein or modulates the expression of the nucleic acid encoding either the BCNG protein or the BCNG-related protein.

In addition, the present invention further provides a method for identifying a compound capable of interacting with a BCNG protein or a BCNG-related protein which

comprises: (a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound under conditions permissive to formation of a complex between the compound and the BCNG protein or the BCNG-related protein; (b) determining the amount of complex formed between the compound and the BCNG protein or the BCNG-related protein; (c) comparing the amount of complex formed in step (b) with the amount formed in the absence of the compound, a greater amount of complex formed in the presence of the compound indicating the presence of a compound capable of interacting with a BCNG protein or a BCNG-related protein.

Also, the present invention provides a method for identifying a compound capable of modulating BCNG protein or BCNG-related protein activity which comprises: (a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound; (b) determining the amount of activity of the BCNG protein or BCNG-related protein in the sample; and (c) comparing the amount of activity of the BCNG protein or the BCNG-related protein determined in step (b) with the amount determined in the absence of the compound, an increase or decrease in activity indicating the presence of a compound capable of modulating the activity of the BCNG protein or the BCNG-related protein.

The present invention also provides for compounds or compositions which are identified through the compound screening methods described herein, as capable of modulating the activity or expression of BCNG protein or BCNG related protein.

An embodiment of this invention is step (a) comprising first introducing the nucleic acid encoding a BCNG

protein or a BCNG-related protein into an expression system and causing the expression system to express the nucleic acid under conditions whereby a BCNG protein or a BCNG-related protein is produced. Another embodiment of this invention is wherein step (b) comprises measuring the channel electrical current or intracellular calcium level in the presence of the compound. In yet another embodiment of this invention, the expression system comprises a cultured host cell.

In an embodiment of this invention, the BCNG protein comprises the amino acid sequence of mBCNG-1 (Seq.ID.No.:2), mBCNG-2 (Seq.ID.No.:6), mBCNG-3 (Seq.ID.No.:10), mBCNG-4 (Seq.ID.No.:12), hBCNG-1 (Seq.ID.No.:4) or hBCNG-2 (Seq.ID.No.:8) or a portion thereof. In one embodiment of the present invention, the sample comprises a cell, cell lysate or cell-free translation. In another embodiment, the cell is a cardiac cell, a kidney cell, a hepatic cell, an airway epithelial cell, a muscle cell, a neuronal cell, a glial cell, a microglial cell, an endothelial cell, a mononuclear cell, a tumor cell, a mammalian cell, an insect cell, or a *Xenopus* oocyte.

The present invention further provides a compound, previously unknown, identified by the screening methods herein. According to one embodiment, the compound is a peptide, a peptidomimetic, a nucleic acid, a polymer, or a small molecule. The small molecule may be an organic or an inorganic molecule. The small molecule may have a molecular weight less than that of a BCNG protein. According to an embodiment of the present invention, the compound is bound to a solid support. In one embodiment of the present invention, the BCNG protein or the BCNG-related protein is ion channel protein or a protein which

is a subunit of an ion channel subunit protein. In one embodiment the BCNG-related protein is a component which is needed to create a pacemaker current in and among cells. In an embodiment of the present invention, the compound is an agonist or antagonist of ion channel activity.

According to an embodiment of the present invention, the modulation is increased ion flow rate or decreased ion flow rate. According to another embodiment, the modulation is increased ion permissivity or decreased ion permissivity.

The present invention also further provides a method of modulating BCNG protein activity or BCNG-related protein activity in a sample, comprising contacting the sample with the provided compound.

Further, the present invention also provides a method of treating a condition in a subject which comprises administering to the subject an amount of the provided compound, effective to treat the condition. The condition comprises an abnormal condition. The abnormal condition may be a loss of memory, a cardiac condition, a hepatic condition, a problem with cellular secretions, a pancreatic condition, a pacemaker condition in brain, or a pacemaker condition in non-neuronal cells.

The present invention additionally provides a pharmaceutical composition which comprises the provided compound and a pharmaceutically acceptable carrier. In an embodiment of this invention, the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, a nonaqueous solution or a solid carrier.

The present invention also additionally provides a method for treating a condition in a subject which comprises administering to the subject an amount of the provided pharmaceutical composition, effective to treat the condition in the subject.

In an embodiment of the present invention, the condition is a neurological, renal, pulmonary, hepatic, or cardiovascular condition. According to an embodiment of this invention, the condition is epilepsy, Alzheimer's Disease, Parkinson's Disease, long QT syndrome, sick sinus syndrome, age-related memory loss, cystic fibrosis, sudden death syndrome, hyperalgesia, ventricular or atrial arrhythmias, familial sinus node disease or a pacemaker rhythm dysfunction. In a further embodiment of this invention, the subject is a human. Certain additional methods for treating diseases in a subject are discussed hereinbelow. Long QT disease is a cardiac disease wherein action potentials last longer than they normally should. Sick sinus disease is an acquired disease (e.g. after atrial fibrillation) wherein the sinus node does not function normally.

The present invention additionally also provides an antibody which binds specifically to a BCNG protein or a BCNG-related protein. The present invention further provides a cell capable of producing the antibody. The present invention also provides a method of identifying a BCNG protein or a BCNG related protein in a sample comprising: a) contacting the sample with an antibody under conditions permissive to the formation of a complex between the antibody and the protein; b) determining the amount of complex formed; and c) comparing the amount of complex formed in step (b) with the amount of complex formed in the absence of the antibody, the presence of an

increased amount of complex formed in the presence of the antibody indicating identification of the protein in the sample.

As used herein, the term "BCNG protein" encompasses a protein having an amino acid sequence substantially similar to or identical to mBCNG-1, mBCNG-2, mBCNG-3, mBCNG-4, hBCNG-1 or hBCNG-2. An example of a BCNG protein is mBCNG-1, mBCNG-2, mBCNG-3, mBCNG-4, hBCNG-1 or hBCNG-2. A BCNG protein may be a homolog of mBCNG-1, mBCNG-2, mBCNG-3, mBCNG-4, hBCNG-1 or hBCNG-2 in a species other than mouse or human. Alternatively a BCNG protein may be another member of the family of BCNG proteins in mouse, human or other mammalian or non-mammalian species. A BCNG protein may function as an integral component or subunit of an ion channel. A BCNG protein may be an accessory protein or a non-functional protein associated with an ion channel.

The term "BCNG-related protein" encompasses a protein having substantial homology to at least one functional domain of a BCNG protein as described herein. (See, Example 3, Figure 11 and Figure 13). For example, the hydrophobic core is one such domain (See, Example 3, subsection "The Hydrophobic Core"). Another example of a functional domain is the S4 voltage-sensing domain (See, Example 3, subsection "The S4 voltage-sensing domain). Still another example of a functional domain is the cyclic nucleotide binding site (See, Example 3, subsection "The cyclic nucleotide binding site). Yet another example is the pore domain ((See, Example 3, subsection "The pore"). A BCNG-related protein may thus function as an integral component or subunit of an ion channel. A BCNG-related protein may be an accessory protein or a non-functional protein associated with an ion channel. A BCNG-related protein is defined by a sequence or structural homology with a BCNG protein or

portion thereof. Thus, a BCNG protein is a BCNG-related protein, but a BCNG-related protein is not limited to BCNG proteins.

The amino acid sequence of mBCNG-1 is presented as Seq.ID.No.: 2. This sequence has been deposited in the GenBank database and accorded the GenBank Accession Number:AF028737.

A BCNG protein exhibits substantial sequence similarity to mBCNG-1. A BCNG-related protein exhibits substantial homology or functional relatedness to mBCNG-1. Substantial sequence homology includes consideration to conserved amino acid substitutions as understood by one of skill in the art. Functional relatedness may be gleaned from domains or regions of sequence having similarity, separated by regions with no apparent homology.

The present invention provides a composition comprising a BCNG protein or portion thereof, a BCNG-related protein or portion thereof, a nucleic acid encoding a BCNG protein or portion thereof, a nucleic acid encoding a BCNG related protein or portion thereof, an antibody to a BCNG protein or portion thereof, an antibody to a BCNG related protein or portion thereof, an nucleic acid with a sequence antisense to a portion of either a BCNG protein or a BCNG related protein or any other described compounds of the present invention. The composition may further comprise a carrier. The carrier may be a pharmaceutically acceptable carrier.

As used herein, a "portion thereof" is a sequence (e.g. am amino acid sequence or a nucleotide sequence) which comprises less than the entire sequence. For example, in reference to Seq.ID.No.: 2, amino acids 35-45 represent a portion thereof.

As used herein, the term "specifically hybridize" means that a nucleic acid probe hybridizes to a nucleic acid sequence having substantial homology with that of the probe. The sequence need not be identical or unique. However, the sequence must indicate a structural or functional relationship between the sequences as is understood in the art.

Hybridization can distinguish between closely-related and distantly-related members of a gene family. Reaction conditions can be adjusted to optimize hybridization of one species and minimize hybridization of others.

For a poorly-matched hybrid, the hybridization is lower and the hybridization curve is displaced towards lower temperatures. When the ratio of rate constants (discrimination ratio) for cross-hybridization and for self-hybridization is plotted against temperature of reaction, a sigmoidal curve is obtained. At low temperatures, the ratio is high while at higher temperatures (approaching T_m -20°C for perfectly-matched hybrids), the ratio approaches zero. The relationship is useful in that it predicts that it should be easier to distinguish between distantly related sequences by incubating at low temperatures while it should be easier to distinguish closely related sequences by hybridizing at high temperatures.

In order, to distinguish between the distantly-related members of a family of sequences, hybridization should take place at a more permissive (relaxed) criterion. To detect closely-related members, the hybridization should be at a stringent criterion. A single compromise criterion will not be effective because, different members of the family probably have different discrimination versus temperature curves. Hybridization at a relaxed criterion followed by washing under

progressively more stringent conditions may be useful for detecting distantly-related members of a family, but is not suitable for identifying closely-related members. This is probably because hybridization and washing depend on different parameters. Hybridization depends on the nucleation frequency while washing depends on the thermal stability (T_M) of the hybrids. Thus, a stringent hybridization followed by a stringent wash is better for detecting closely-related members of a family than permissive hybridization and a stringent wash.

The degree of hybridization depends on the degree of complementarity, the length of the nucleic acid molecules being hybridized, and the stringency of the conditions in a reaction mixture. Stringency conditions are affected by a variety of factors including, but not limited to temperature, salt concentration, concentration of the nucleic acids, length of the nucleic acids, sequence of the nucleic acids and viscosity of the reaction mixture. More stringent conditions require greater complementarity between the nucleic acids in order to achieve effective hybridization.

A preferred method of hybridization is blot hybridization. See Sambrook et al. 1989 *Molecular Cloning: A Laboratory Manual* 2nd Ed. for additional details regarding blot hybridization.

As used herein, a nucleic acid probe is a nucleic acid which specifically hybridizes to a particular nucleic acid sequence. The probe may be bound nonspecifically to a solid matrix. The nucleic acid probe may be DNA or RNA and can be labeled with a detectable marker. Such labeling techniques methods include, but are not limited to, radio-labeling, digoxygenin-labeling, and biotin-labeling. A well-known method of labeling DNA is ^{32}P using DNA polymerase, Klenow enzyme or polynucleotide

kinase. In addition, there are known non-radioactive techniques for signal amplification including methods for attaching chemical moieties to pyrimidine and purine rings (Dale, R.N.K. et al., 1973 *Proc. Natl. Acad. Sci. USA* 70:2238-42), methods which allow detection by chemiluminescence (Barton, S.K. et al., 1992 *J. Am. Chem. Soc.* 114:8736-40) and methods utilizing biotinylated nucleic acid probes (Johnson, T.K. et al., 1983 *Anal. Biochem.* 133:125-131; Erickson, P.F. et al., 1982 *J. Immunol. Methods* 51:241-49; Matthaei, F.S. et al., 1986 *Anal. Biochem.* 157-123-28) and methods which allow detection by fluorescence using commercially available products. Non-radioactive labeling kits are also commercially available.

Nucleic acid amplification is described in Mullis, U.S. Patent No. 4,683,202, which is incorporated herein by reference.

In a polymerase chain reaction (PCR), an amplification reaction uses a template nucleic acid contained in a sample can use one or more probe ("primer") sequences and inducing agents.

Suitable enzymes to effect amplification, specifically extension include, for example, *E.coli* DNA polymerase I, thermostable *Taq* DNA polymerase, Klenow fragment of *E.coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase and other enzymes which will facilitate covalent linkage of the nucleotides to polynucleotides which are form amplification products. Oligonucleotide probes (primers) can be synthesized by automated instruments sold by a variety of manufacturers or can be commercially prepared.

Solid matrices are available to the skilled artisan. A

solid matrix may include polystyrene, polyethylene, polypropylene, polycarbonate, or any solid plastic material in the shape of test tubes, beads, microparticles, dip-sticks, plates or the like. Additionally matrices include, but are not limited to membranes, 96-well microtiter plates, test tubes and Eppendorf tubes. Solid phases also include glass beads, glass test tubes and any other appropriate shape made of glass. A functionalized solid phase such as plastic or glass which has been modified so that the surface carries carboxyl, amino, hydrazide, or aldehyde groups can also be used. In general such matrices comprise any surface wherein a ligand-binding agent can be attached or a surface which itself provides a ligand attachment site.

As used herein, the term "modulation" in reference to modulation of protein activity or ion channel activity refers to the up-regulation or down-regulation of the activity. Up-regulation includes, but is not limited to increased ion flow in the case of an ion channel. Down-regulation includes, but is not limited to decreased ion flow in the case of an ion channel. For example, one form of modulation of activity would be a channel-blocking protein or compound which inhibits ion flow through the channel, decreasing activity. Alternatively, another form of modulation is a channel-opening protein or compound which facilitates flow through the channel, increasing activity. In addition, the nature of the ion flow through a channel may be modulated. For example, proteins or compounds may alter a channel refractive to potassium flow to become permissive to potassium flow in addition to or in place of another ion. The term modulation is also used to describe the increase or decrease of gene expression.

In the practice of any of the methods of the invention or

in the preparation of any of the pharmaceutical compositions of the present invention a "therapeutically effective amount" is an amount which is capable of modulating the activity or function of a BCNG-related protein. Accordingly, the effective amount will vary with the subject being treated, as well as the condition to be treated. The methods of administration may include, but are not limited to, administration cutaneously, subcutaneously, intravenously, parenterally, orally, topically, or by aerosol.

As used herein, the term "suitable pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

This invention also provides for pharmaceutical compositions together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl.,

acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the compound, complexation with metal ions, or incorporation of the compound into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, micro emulsions, micelles, unilamellar or multi lamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the compound or composition.

Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

When administered, compounds are often cleared rapidly

from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses of bioactive compounds may by required to sustain therapeutic efficacy. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-compound adducts less frequently or in lower doses than with the unmodified compound.

Attachment of polyethylene glycol (PEG) to compounds is particularly useful because PEG has very low toxicity in mammals (Carpenter et al., 1971). For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treatment of severe combined immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicity and antigenicity of heterologous compounds. For example, a PEG adduct of a human protein might be useful for the treatment of disease in other mammalian species without the risk of triggering a severe immune response. The carrier

includes a microencapsulation device so as to reduce or prevent an host immune response against the compound or against cells which may produce the compound. The compound of the present invention may also be delivered microencapsulated in a membrane, such as a liposome.

Polymers such as PEG may be conveniently attached to one or more reactive amino acid residues in a protein such as the alpha-amino group of the amino terminal amino acid, the epsilon amino groups of lysine side chains, the sulfhydryl groups of cysteine side chains, the carboxyl groups of aspartyl and glutamyl side chains, the alpha-carboxyl group of the carboxy-terminal amino acid, tyrosine side chains, or to activated derivatives of glycosyl chains attached to certain asparagine, serine or threonine residues.

Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonate. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification of protein free sulfhydryl groups. Likewise, PEG reagents containing amino hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

The invention provides nucleic acids comprising cDNA encoding BCNG protein as listed below:

Plasmid name	ATCC Accession No.	Date of Deposit
mBCNG-1	209781	April 21, 1998
mBCNG-2a	209825	May 1, 1998
mBCNG-2b	209826	May 1, 1998
mBCNG-3a	209824	May 1, 1998
mBCNG-3b	209828	May 1, 1998
hBCNG-1	209827	May 1, 1998
hBCNG-2	209829	May 1, 1998

The above-identified plasmids, provided by the present invention, were deposited with The American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20108-0971, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

Treatment of Diseases or Conditions in a Subject

The compounds and compositions of the present invention may be administered to a subject to treat a disease or condition which is associated with pacemaker function. The compounds and compositions comprises not only BCNG proteins or BCNG related proteins and nucleic acids encoding the same or portions thereof, but also compounds identified by the screening methods of the present invention. For example, a compound useful in the present invention may be a peptide, a small molecule (organic or inorganic), a peptidomimetic, or other compound as described hereinabove.

For example, the compounds and compositions of the present invention may be useful for treating memory deficits or disorders. Such memory disorders or deficits may involve an abnormal pacemaking function in the cells

of the brain and the central nervous system. The memory disorder or deficit may be due to Alzheimer's disease, Parkinsons's Disease, or age-related memory loss.

The present invention provides for a method for treating a sensory disorder in a subject comprising administering to the subject compounds or compositions of the present invention. Such sensory disorders include sensory disorders of the eyes (blindness, loss of vision), of the nose (loss of smell), touch (numbness), and taste (lack of ability to taste).

In another example, the present invention provides for a method for treating a subject suffering from an auditory disorder which comprises administering an amount of the compounds or compositions of the present invention. It is shown hereinbelow that BCNG isoforms are expressed in the tissues of the auditory system in significant amounts. It may be possible to change the response characteristics of the cells of the auditory systems by regulation of the BCNG genes in these auditory tissues and cells. Thus, administration of nucleic acids or proteins (for example via a localized virus vector, or via a liposome carrying protein) to the subject would treat such an auditory disorder. The auditory disorder may be deafness or loss of hearing.

In another embodiment, the present invention provides for a method for treating a subject suffering from a respiratory disorder either due to defects in CNS (central nervous system) areas that control respiration or due to defects in the lung, which comprises administering to the subject an amount of a compound or composition of the present invention. Preferably, the compound comprises the BCNG isoform or a compound (e.g.

a small molecule) which interacts with the BCNG isoform, which is normally expressed in lung tissue and/or brain nuclei important for respiratory control. The respiratory disorder may be Sudden Infant Death Syndrome, or any difficulty in regular breathing (e.g. shortness of breath). The respiratory disorder may be asthma.

The present invention provides a method for the treatment of dyslexia in a subject which comprises administering to the subject a compound or composition of the present invention in an amount effective to treat the dyslexia in the subject. The present invention also provides methods for treatment of attention deficit disorder or learning disabilities. Learning related disorders may result from abnormal functioning (either increased or decreased) of ion channels present in the thalamus. This region of the brain is considered to be involved in wakefulness, attention and arousal. Disorders involving abnormal states of such functions may be treatable using the compounds and pharmaceutical compositions of the present invention.

The present invention provides a method for treating symptoms of drug addiction in a subject which comprises administering to the subject a composition of the present invention to thereby modulate ion channel function in the subject and treat the symptoms of drug addiction in the subject.

The present invention provides a method for regulating the secretions of a cell which normally produces secretions in a subject suffering from abnormal secretions or lack of secretions which comprises administering to the subject a therapeutically effective amount of a compound or composition of the present

invention in order to regulate the secretions of the cell. The compound or composition may regulate the resting phase or the secretion phase of the cell so as to regulate when the cell produces secretions. The cell may be a pancreatic cell, a liver cell or a spleen cell.

The present invention provides a method for regulating rebound excitation in non-pacemaking cells.

The BCNG proteins are useful targets for screens for drugs that are effective in the control of pain and hyperalgesia. Pacemaker type channels with properties similar to those of the expressed BCNG-1 protein have been identified in primary afferent sensory neurons, where the channels are activated by prostaglandin E2, a hyperalgesia-inducing agent released during inflammation (Ingram and Williams, 1996). The channels have been proposed to play a role in pain perception and hypersensitivity to painful stimuli. The present invention provides a method for treating pain in a subject which comprises administering to the subject an amount of the composition of the present invention

The BCNG channel isoforms expressed in cardiac ventricular muscle, including BCNG-2, are useful targets for screens for drugs that are effective in treating ventricular and/or atrial arrhythmias due to abnormal pacemaker activity in these tissues. Pacemaker channels with abnormal activation properties are detected in non-pacemaking regions of the heart, including ventricle, during heart failure (Cerbai et al., 1994, 1997).

The BCNG gene isoforms expressed in sinoatrial node can provide a useful genetic screen for inherited diseases of cardiac pacemaker function such as familial sinus node

disease. Certain familial, inherited cardiac diseases are thought to involve defects in pacemaker channel function in the sinoatrial node (Spellberg, 1971).

The BCNG isoforms expressed in heart will be useful to screen for improved drugs that can limit heart muscle damage during episodes of ischemia. Pacemaker channel blockade with the compound ZD 7288 has been shown to reduce infarct size during myocardial ischemia (Schlack et al., 1998).

The BCNG-1 channel isoforms will be useful as a screen for drugs that alter pancreatic function, including compounds that stimulate or inhibit insulin release. It was demonstrated that the human BCNG-1 protein is expressed in pancreas (Santoro et al., 1998).

The BCNG channel isoforms will be useful to screen drugs that alter function of kidney and liver. BCNG isoforms are expressed in these tissues where they could contribute to hormone release and ion transport functions. Cyclic nucleotide (cGMP) -stimulated activity of a 1 pS channel, similar to the conductance of the pacemaker channel, has been reported for renal epithelial cells (Marunada et al, 1991). Liver cells have been shown to exhibit cation permeable channels coupled to cellular metabolism (Lidofsky et al. 1997).

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Example 1: Interactive cloning with the SH3 domain of N-src identifies a new brain-specific ion channel protein, with homology to cyclic nucleotide-gated channels

By screening for molecules that interact with the neuronal form of Src tyrosine kinase a novel cDNA was isolated that appears to represent a new class of ion channels. The encoded polypeptide, mBCNG-1, is distantly related to proteins in the family of the cyclic nucleotide-gated channels and the voltage-gated channels, Eag and H-erg. mBCNG-1 is expressed exclusively in the brain as a glycosylated protein of approximately 132 kD. Immunohistochemical analysis indicates that mBCNG-1 is preferentially expressed in specific subsets of neurons in the neocortex, hippocampus and cerebellum, in particular pyramidal neurons and basket cells. Within individual neurons, the mBCNG-1 protein is localized to either the dendrites or the axon terminals depending on the cell type.

Southern blot analysis shows that several other BCNG-related sequences are present in the mouse genome, indicating the emergence of an entirely new subfamily of ion channel coding genes. These findings suggest the existence of a novel class of ion channel, which is potentially able to modulate membrane excitability in the brain and which may respond to regulation by cyclic nucleotides.

Defining signal transduction pathways that contribute to the control of synaptic strength in the brain is an

important and long-sought goal. In an effort to identify the biochemical targets of Src-family tyrosine kinases in the central nervous system, the yeast two- hybrid system was used to clone proteins that could interact with the SH3 domain of the neural specific form of Src kinase (Brugge, et al., 1985; Martinez, et al., 1987). As a result of this screening a new protein, mBCNG-1 (mouse Brain Cyclic Nucleotide Gated-1) was identified and isolated.

mBCNG-1 has been identified and characterized as an ion channel protein and exhibits sequence homology to voltage-gated potassium channels, CNG channels, and plant inward rectifiers. Southern blot analysis suggests that this is the first member of a new family of proteins. mBCNG-1 is expressed exclusively in the brain and is preferentially localized to the processes of subsets of neurons in the neocortex, cerebellar cortex and hippocampus. The specific localization pattern of mBCNG-1 and the potential for a direct interaction with cyclic nucleotides suggest that it may represent a new brain-specific ion channel protein that is an important component in the expression of intercellular and intracellular signaling.

Results

Isolation of mBCNG-1. mBCNG-1, a novel cDNA with homology to CNG-related and Eag-related ion channels was initially isolated and identified by interactive cloning with the N-src SH3 domain in a yeast two-hybrid screen. The src gene expresses an alternatively spliced form (N-src or $pp60^{src-c(+)}$, which is specific for neuronal cells and has an increased kinase activity (Brugge, et al., 1985). The N-src protein differs from the non-neuronal form (c-src or $pp60^{src-c}$) by an insertion of

six amino acids in the region corresponding to the Src homology 3 (SH3) domain of the protein (Martinez et al. 1987). SH3 domains are considered modules for protein-protein interaction (Pawson, et al., 1995). Therefore the yeast two-hybrid screen (Fields, et al., 1989; Zervos, et al., 1993) was used to identify brain specific proteins that would selectively interact with the N-src SH3 domain.

The screening of 5 x 10^5 independent clones with the N-src SH3 bait resulted in the isolation of a single positively reacting fusion product (pJG-d5). This clone encoded a protein that showed a strong interaction with the N-src SH3 domain, but no significant interaction with the c-src, fyn, or abl SH3 domains, indicating a specific recognition of the N-src SH3 domain in the yeast two-hybrid system. The sequence analysis of pJG-d5 indicated that it encodes the C-terminal portion of a larger protein. Overlapping cDNA clones were therefore isolated from a λgt10 library and an open reading frame (ORF) was identified that encodes a 910 amino acid polypeptide with a predicted molecular weight of 104 kDa (Fig. 1A). The pJG-d5 insert corresponds to its C-terminal amino acids 404-910.

The N-terminal part of the predicted protein contains an hydrophobic core comprising seven hydrophobic domains (Fig. 1B). These domains show significant homology to the six transmembrane domains (S1-S6) and the pore region (P) of voltage activated K^+ channels (Fig. 1C). In addition to the hydrophobic core, there is a putative cyclic nucleotide binding site (CNBs) in the C-terminal half of the protein (amino acids 472-602, Fig. 1A and Fig. 1D). This cyclic nucleotide binding

site is most closely related to the corresponding region in cyclic-nucleotide gated channels (30% similarity). The amino acids that lie close to the bound cyclic nucleotide in the bacterial catabolite gene activator protein (CAP) are conserved in the N-src interacting protein, suggesting that the CNBs is functional (Weber et al., 1989). On the basis of these features, the newly identified protein was designated mBCNG-1 (mouse Brain Cyclic Nucleotide Gated-1).

Among all the known K^+ channel superfamily genes, the core region of mBCNG-1 displays the highest amino acid similarity (22%) to the corresponding region in the mouse Eag protein, whereas the sequence similarity to cyclic nucleotide-gated channels is only 17% in this region (distances were determined by the MegAlign program of DNASTAR). The S4 domain of mBCNG-1 has a total of eight positively charged residues (two groups of four, separated by a serine), which again makes it more similar to voltage activated K^+ channels (*Sh* and *eag* families) than to cyclic nucleotide-gated channels.

The putative pore forming region of the mBCNG-1 protein (Figure 1C) is also most closely related to the corresponding region in Shaker and Eag-related channels (30% sequence similarity in either case). However, it contains significant substitutions in two positions that are otherwise highly conserved in voltage activated K^+ channels: the aspartate residue which follows the GYG triplet is replaced with alanine (position 352) and the serine/threonine residue at -8 from that position is replaced with histidine (position 344). Similar substitutions are

found in the β-subunit of the retinal CNG-channel, where the position corresponding to the aspartate is occupied by a leucine and a lysine is found at -8 from that position (Chen, et al., 1993). This suggested that the mBCNG-1 protein might be incapable of conducting current *per se*, but may act in combination with a second not yet identified polypeptide to form a functional heteromultimeric ion channel.

mBCNG-1 is a 132 kDa Glycoprotein. To characterize the protein encoded by the mBCNG-1 cDNA (ATCC Accession No. 209781) (Seq.ID.No.: 1), antibodies were generated against two separate domains in the predicted cytoplasmic tail: amino acids 594-720 (fusion protein GST-q1; antiserum αq1) and amino acids 777-910 (fusion protein GST-q2; antiserum αq2). Both antisera specifically immunoprecipitated the *in vitro* translation product of the cloned mBCNG-1 sequence.

In Western blots of mouse brain extracts, both the αq1 and αq2 antisera recognized a diffuse band with an apparent molecular mass of 132kDa (Fig. 2A). Complete abolition of the labeling by preadsorbing the antisera with a GST-fusion protein incorporating both antigenic domains (GST-d5, amino acids 404-910) indicates it represents the native mBCNG-1 subunit. Treatment of the brain extract with N-glycosidase F prior to the Western blotting results in a substantial reduction of the molecular weight of the observed band, which now co-migrates with the *in vitro* translated BCNG-1 product (Fig. 2B).

Sequence analysis indicates that three N-glycosylation consensus sites are present in the mBCNG-1 protein. Among these, Asn 327 is predicted to lie between

transmembrane domain S5 and the pore (P) on the extracellular side of the plasma membrane (Fig. 1A and Fig. 1B). This site corresponds to Asn 327 of the cGMP-gated channel from bovine rod photoreceptors, where it has been demonstrated to be the sole site of glycosylation (Wohlfart et al., 1992). Together, these data suggested that the cloned cDNA sequence encodes the full length product of the mBCNG-1 gene and that mBCNG-1 is a N-linked glycoprotein.

mBCNG-1 is expressed in neurons. Northern blot analysis revealed the presence of multiple mBCNG-1 transcripts in poly(A)$^+$ RNA from the brain, the most abundant species being 3.4, 4.4, 5.8 and 8.2 kb long (Figure 3). The 3.4 kb transcript corresponds in size to the cloned cDNA. No expression was detected in the heart, spleen, lung, liver, skeletal muscle, kidney or testis. The specific expression of the mBCNG-1 protein was confirmed by Western blot analysis.

The cellular localization of mBCNG-1 within the brain was examined by *in situ* hybridization (Fig. 4) and by immunohistochemical staining (Figs. 5A-5F). In both cases, the highest levels of mBCNG-1 expression were detected in the cerebral cortex, in the hippocampus, and in the cerebellum.

In the cerebral cortex, *in situ* hybridization shows a strong expression of the mBCNG-1 mRNA layer V pyramidal neuron cell bodies that are distributed in a continuous line along the neocortex (Fig. 4). Immunohistochemical analysis reveals a strict subcellular localization of the mBCNG-1 protein within these cells. Staining of the apical dendrites (Fig. 5A) extends into the terminal branches of these

fibers and is particularly intense in layer I, which contains the terminal dendritic plexus of the pyramidal neurons (Fig.5B).

A similar expression pattern can be recognized in the hippocampus. Here, the *in situ* hybridization shows a strong mBCNG-1 mRNA expression in the pyramidal cell body layer of areas CA1 and CA3 (Fig.4). The labeling in area CA3 is somewhat less prominent than the labeling in area CA1. At the protein level, the most intense mBCNG-1 immunostaining is observed along the hippocampal fissure, in the layer corresponding to the *stratum lacunosum-moleculare* (Fig. 5C). This layer contains the terminal branches of the apical dendrites of the pyramidal neurons in area CA1 (Raisman, 1965). Further mBCNG-1 immunoreactivity is detected within the *stratum pyramidale* of areas CA1 and CA3; the staining, however, is absent from the pyramidal cell bodies but is rather present in the fibers surrounding them (Fig. 5D). These fibers most likely represent the basket cell plexus associated to pyramidal neurons.

The immunostaining in the cerebellum also shows a pattern characteristic of basket cell expression. In the cerebellar cortex, basket cell nerve endings branch and contact the initial segment of the Purkinje cell axon in a distinct structure known as "pinceau" (Palay, et al., 1974). As shown in Figures 5E and 5F, these structures are intensely labeled by the αq1 and αq2 antisera, while the staining excludes the Purkinje cell bodies. Thus, in basket cells, the mBCNG-1 protein appears to be selectively localized to axons and is particularly enriched in the nerve terminals. An intense labeling of some brainstem nuclei

is observed by in situ hybridization (Fig. 4) and areas of immunoreactivity were detected in other brain regions, including the olfactory bulb.

mBCNG-1 defines a new subfamily of K^+ channel genes. Most of the ion channel sequences characterized so far are members of evolutionarily related multigene families. To investigate whether more sequences related to mBCNG-1 exist, mouse genomic DNA Southern blots were analyzed under various stringency conditions (Figure 6).

The probe (B1-T) was designed in the hydrophobic core region of mBCNG-1, including transmembrane domains S5, P and S6; the repeat region in the C-terminal portion of the protein was excluded. Reducing the stringency of the hybridization conditions from 8°C below the melting temperature of the B1-T probe (Figure 6A) to 33°C below the melting temperature (Figure 6B) resulted in the detection of a number of additional hybridization signals in every lane of the blot. None of the known sequences in the K^+ channel superfamily has sufficient homology to mBCNG-1 to hybridize under these conditions. This result suggested that mBCNG-1 is the first known member of a larger group of related genes, which represent a new branch in the voltage-gated K^+ channel superfamily.

Discussion

Voltage-gated potassium (VGK) channels constitute a large and still expanding superfamily of related genes (Strong, et al., 1993; Warmke and Gonetzky, 1994). The most widely used strategy for cloning new genes in the VGK family has been by homology to a small number of initial members (Sh, eag, and slo from

Drosophila (Papazian, et al., 1987; Kamb, et al., 1987; Warmke, et al., 1991; Atkinson, et al., 1991); cGMP-channel from bovine retina (Kaupp, et al., 1989). Unfortunately, this approach is not well suited for identifying more divergent sequences. Expression cloning in *Xenopus* oocytes can circumvent this problem, however, this implies a pre-existing or readily detectable physiological characterization of the channel.

An alternative cloning strategy that requires no *a priori* knowledge of the structure or activity of the target protein is to screen for K^+ channels by means of protein-protein interactions. Using the SH3 domain of N-src as a bait, a protein, mBCNG-1, was obtained that appears to constitute a new branch of the K^+ channel superfamily. mBCNG-1 displays the motifs of a voltage-gated K^+ channel (six transmembrane spanning domains, a highly basic S4, and a P region) (Strong, et al., 1993; Warmke, et al., 1994 and Figs. 1A-1D). mBCNG-1, despite its similarity to voltage activated K^+ channel superfamily members, with defined by the presence of six transmembrane domains and a pore-like region (Warmke, et al., 1994), shows considerable divergence from all of the other known sequences. Although the cyclic nucleotide binding site of mBCNG-1 is most similar to the site present in CNG channels (30%), the S4 and previous are most closely related to the corresponding regions in *Shaker* and *Eag*. Overall, the highest similarity in the hydrophobic core region is to mouse Eag Protein (22%). Thus, mBCNG-1 appears to constitute a new branch of the K^+ channel superfamily.

The fusion between an ancestral K^+ channel and an ancestral cyclic nucleotide binding site is likely to have occurred prior to the evolutionary separation

between plants and animals (Warmke, et al., 1994). Divergence from this common ancestor would have led on one hand to Eag-related channels and plant inward rectifiers (which maintained more of the features of voltage activated K^+ channels, while showing a progressive deviation from the original CNBs sequence) and on the other hand to CNG-channels (which show a higher evolutionary constraint on the cyclic nucleotide binding site, while they have lost voltage activation and K^+ selectivity). The features of mBCNG-1 suggest that it may have remained closer to the ancestral molecule that represents the evolutionary link between voltage-gated K^+ channels and cyclic nucleotide-gated channels.

The emerging pattern for olfactory and retinal CNG-channels and the non-consensus sequence of the putative pore forming region of mBCNG-1 suggests that the lack of detectable electric current following mBCNG-1 expression in xenopus oocytes is due to mBCNG-1 representing a β subunit of a heteromultimeric channel (Chen, et al., 1993; Liman, et al., 1994; Bradley, et al., 1994). Indeed the data show the existence of a number of BCNG-related sequences in the mouse genome, and one or more of these genes could encode additional subunits required for the formation of an active channel.

mBCNG-1 protein is expressed only in the brain and in particular in two of the principal classes of neurons within the cerebral, hippocampal and cerebellar cortexes: pyramidal neurons and basket cells. This distribution would be consistent with an *in vivo* interaction of mBCNG-1 with N-src, which is also expressed in cerebral and hippocampal pyramidal neurons

(Sugrue, et al., 1990). The observed interaction between mBCNG-1 and the N-src SH3 domain is intriguing as is its physiological relevance and the role of the proline-rich region. The possibility that other factors may target the proline-rich region of mBCNG-1 has also to be considered, particularly in view of the recently discovered WW domains (Sudol, et al., 1996; Staub, et al., 1996).

The varied subcellular localization of mBCNG-1 (dendritic in pyramidal cells and axonal in basket cells) suggests that mBCNG-1 could play different roles in different populations of neurons, perhaps by regulating presynaptic or postsynaptic membrane excitability depending on the cell type. A similar distribution has been demonstrated for the K^+ channel subunit Kv 1.2 (Sheng, et al., 1994; Wang, et al., 1994). Kv 1.2 forms heteromultimeric K^+ channels with several other *Shaker* type subunits, which have an overlapping yet differential pattern of expression, giving rise to a range of conductances with diversified functional characteristics.

The presence of mBCNG-1 in the dendrites of hippocampal pyramidal cells is particularly intriguing; cAMP has been shown to be important for the establishment of some forms of long-term synaptic potentiation in these cells (Frey, et al., 1993, Bolshakov, et al., 1997; Thomas, et al., 1996). The structural features of mBCNG-1 predict a K+ conducting activity, directly modulated by cyclic nucleotide binding. Interestingly, a current with similar characteristics has been described in the hippocampal pyramidal neurons of area CA_1 (Pedarzani, et al., 1995), where mBCNG-1 is highly expressed. This current (I_Q) is believed to contribute to the noradrenergic

modulation of hippocampal activity, by regulating neuronal excitability in response to cAMP levels. mBCNG-1 could participate in the formation of the channels responsible for this type of current.

Experimental Procedures

Yeast two hybrid interaction cloning of mBCNG-1. The two-hybrid screen was performed following published procedures (Zervos, et al., 1993); the reagents used included plasmids pEG202, pJG4-5, pJK103 and *Saccharomyces cerevisiae* strain EGY48 (*MATa trp1 ura3 his3 LEU2::pLexAop6-LEU2*).

The bait was created by subcloning the SH3 domain of N-src in plasmid pEG202, and contains amino acids 83-147 from the mouse N-src sequence (Martinez, et al., 1987). The cDNA fusion library was constructed in plasmid pJG4-5, using poly(A)$^+$ RNA from the whole brain of an adult C57BL/6 male mouse; the cDNA was synthesized using random hexamers and the GIBCO-BRL SuperScript II synthesis kit, according to the manufacturer's instructions. Only the library constructed from the two fractions with an average cDNA size of > 1.5 kb (total of 1×10^6 independent clones) was used in the two hybrid screen. Library amplification was done in 0.3% SeaPrep agarose (FMC) to avoid changes in complexity.

For library screening, *Saccharomyces cerevisiae* strain EGY48 was first cotransformed with the bait plasmid pEG202-Nsrc and the reporter plasmid pJK103. The resulting strain was maintained under selection for the HIS3 and URA3 markers, and subsequently transformed with the mouse brain cDNA library in plasmid pJG4-5. This description though is more accurate and should be substituted for the transformation mix was grown for two

days in a Ura⁻ His⁻ Trp⁻ -glucose medium containing 0.3% SeaPrep agarose (FMC); the cells were then harvested and plated on Ura⁻ His⁻ Trp⁻ Leu⁻-galactose. Leu⁺ colonies were screened for β-galactosidase activity using a filter lift assay (Breede and Nasmith, 1985). Positively reacting fusion products were isolated and tested for specificity following retransformation into an independent yeast strain. Fusion product pJGd5 corresponds to the C-terminal part of mBCNG-1 (amino acids 404-910; see Figure 8).

Full length cloning of mBCNG-1. For the isolation of the 5' end region of the mBCNG-1 cDNA, two rounds of PCR were performed on the pJG4-5 library, using nested oligonucleotides derived from the pJG-d5 sequence. The downstream primer in the first round was: 5'-AGAGGCATAGTAGCCACCAGTTTCC-3' (Seq. ID. No.: 13) (d5.RL, corresponding to amino acids 456-463 of the mBCNG-1 sequence; see Figure 8). The downstream primer in the second round was: 5'-CCGCTCGAGGCCTTGGTATCGGTGCTCATAG-3' (Seq. ID. No.: 14) (d5.N2), corresponding to amino acids 424-430 of mBCNG-1 and an added XhoI site). The upstream primer was either of two oligonucleotides designed in the pJG4-5 vector sequence: 5'-GAAGCGGATGTTAACGATACCAGCC-3' (Seq. ID. No.: 15) (B42), located 5' to the EcoRI site in the B42 acidic patch, or: 5'-GACAAGCCGACAACCTTGATTGGAG-3' (Seq. ID. No.: 16) (ter), located 3' to the EcoRI site in the ADH terminator.

PCR cycling was performed as follows: 1x(2 minutes, 94°C);
25x(45 seconds, 94°C; 30 seconds, 58°C; 3 minutes, 72°C);
1x(10 minutes, 72°C).

The longest amplification product obtained from this

series of reactions was a 700 bp DNA fragment, which contained amino acids 204-430 from the mBCNG-1 sequence (See Figure 8). This fragment was subcloned, repurified and used as a probe to screen a Mouse Brain cDNA library in λgt10 (CLONTECH, cat. no. ML3000a), in high stringency conditions (hybridization overnight at 65°C in 50% formamide, 5x SSC (1x SSC= 0.15 M sodium chloride/0.015 sodium citrate, pH 7), 5x Denhardt's (1x Denhardt's = 0.02% Ficoll/0.02%polyvinylpirrolidone/0.02% bovine serum albumin), 0.5% SDS, 100 mg/ml salmon sperm DNA. Washing: 10 minutes, room temperature in 2x SSC/0.1% SDS, followed by twice 30 min at 65°C in 0.2x SSC/0.1% SDS.

Positively reacting clones were further screened by PCR, using oligonucleotide d5.RL (Seq. ID. No.: 13) as a downstream primer. The upstream primer was either of the two following vector oligonucleotides: 5'-GAGCAAGTTCAGCCTGGTTAAGTCC-3' (Seq. ID. No.: 17) (15'.N2), located 5' to the EcoRI site in the λgt10 sequence, or 5'-GTGGCTTATGAGTATTTCTTCCAGGG-3' (Seq. ID. No.: 18) (13'.N2), located 3' to the EcoRI site. PCR cycling was performed as described above.

The resulting products were subcloned and sequenced. The longest extension contained amino acids 1-463 of the mBCNG-1 sequence (See Figure 8); the overlapping region of this insert with the insert contained in clone pJG-d5 (amino acids 405-463) includes a Bgl II site, which was used to join the 5' and 3' fragments of the mBCNG-1 cDNA in plasmid pSD64TF for expression studies.

In vitro transcription (MESSAGE MACHINE, Ambion, Austin, TX) and translation *in vitro* Express, Stratagene.

Northern/Southern Blot Hybridization

PCR-generated cDNA fragments corresponding to the indicated amino acids 6-131 (λgt10-derived) 5' sequence) and 594-720 (pJG-5 derived 3[1] sequence) were used to probe a Multiple Tissue Northern Blot (CLONTECH, 7762-1).

For Southern blots, a Mouse Geno-Blot (CLONTECH, 7650-1) was probed using a PCR generated cDNA fragment (B1-T) corresponding to amino acids 270-463 of the mBCNG-1 sequence, as described (Sambrook, 1989). Blots were hybridized at 65° (5x standard saline citrate 1xSSC = 0.15M sodium chloride/0.015 M sodium citrate, pH7 buffer in aqueous solution) and washed as described in figure legends. Washings conditions were as indicated. The melting temperature (TM) for the B1-T probe was calculated according to the formula Tm = 81.5°C + 16.6 (logM) + 0.41 (%GC) - (675/L), where M is the cation concentration and L is the probe length in base pairs.

Antibody production, extracts and Immunochemistry and *in situ* hybridization. The Glutathione S-transferase (GST)-fusion proteins were created by subcloning the q1 (corresponding to amino acids 594-720 of the mBCNG-1 protein) or q2 (corresponding to amino acids 777-910 of the mBCNG-1 protein)(see Fig. 1A) in plasmid pGEX-lombole (Pharmacia), followed by induction and purification of essentially as described (Frangioni and Neel, 1993). Fusion proteins were eluted in phosphate buffered saline (PBS) and injected into rabbits as a 1:1 suspension with Freund adjuvant (Pierce). Antisera were prepared and tested essentially as described (Grant, et al., 1995).

For Western Blot analysis, mouse brain extracts were separated on a 10% SDS-PAGE and electroblotted to PVDF membranes (Immobilon-P, Millipore) as described (Grant, 1995). Blocking and antibody incubations were done in

TBST (10mM Tris pH 7.5, 150mM NaCl, 0.1% Tween-20) + 2% BSA. The αq1 and αq2 antisera were used at a 1:1000 dilution. Secondary anti-rabbit antibodies coupled to alkaline phosphatase (Bio-Rad) were used at a 1:5000 dilution, and the bands were visualized by incubation in NBT\BCIP (Boehringer Mannheim). Total brain extracts were prepared as described (Grant, 1995). For N-glycosidase treatment, 2% SDS was added to the extract and the proteins denatured by boiling for 10 min; reactions were carried out in 50 mM NaP (pH 7.2), 25 mM EDTA, 0.5% Triton-X100, 0.2% SDS, 1 mg/ml protein and 20 U/ml N-glycosidase F (Boehringer) for 1 hr at 37°C.

For immunohistochemistry, 20 μm cryostat sections of mouse brain (fixed in 4% paraformaldehyde/PBS), quenched in 50 mM NH_4Cl/PBS, were blocked (10% goat serum, 0.1% goat serum, 0.1% saponin in PBS) and then exposed to αg1 or αq2 antisera (diluted 1:400 in blocking solution). After washing in PBS + 0.1% saponin, sections were incubated with Cy3-conjugated goat anti-rabbit F(ab') 2 fragments (Jackson Immunoreasearch Labs) diluted 1:200 in blocking solution.

In situ hybridization was performed essentially as described (Mayford et al., 1995) using oligonucleotide probes labeled by 3^1 tailing with using (^{35}S) thio-dATP and terminal transferase (Boehringer Mannheim) to a specific activity of $5x10^8$ cpm/μg. Hybridizations were carried out at 37°C. Slides were washed at 60°C in 0.2x SSC and exposed to film for 2 weeks.

EXAMPLE 2: Identification of a Family of BCNG Genes

Introduction

The original sequence in the BCNG family (mBCNG-1) was

isolated from a mouse brain cDNA library using yeast two-hybrid interaction cloning with the n-Src tyrosine kinase as a bait as described in Example 1. The DNA and amino acid sequences of this protein are Seq. ID. No.:1 and Seq. ID. No.:2 respectively.

Three additional mouse and two human cDNA clones encoding regions homologous to mBCNG-1 (ATCC Accession No. 209781) were isolated. Partial cDNA clones representing two of the mouse genes ([mBCNG-2 [ATCC Accession Nos. 209825 and 209826] and mBCNG-3 [ATCC Accession Nos. 209824 and 209828]) were isolated while screening for full length mBCNG-1 products, and a fourth mouse gene (mBCNG-4) as well as two human genes (hBCNG-1 [ATCC Accession No. 209827] and hBCNG-2 [ATCC Accession No. 209829]) were identified following an EST database homology search, using the protein sequence of mBCNG-1 (Seq.ID.No.: 2) as a query. Further extensions of the identified cDNA clones were subsequently obtained by library screening or RT-PCR cloning. A schematic representation of the mouse and human BCNG sequences identified is presented in Figures 7A-7B.

The three additional mouse proteins described herein below are closely related to each other, having a sequence similarity of 84-88%, but are very distantly related to all other known members of the potassium channel superfamily, including Eag-related channels (22% similarity) and cyclic nucleotide-gated channels (17% similarity).

Northern blot analysis showed individual patterns of tissue distribution for each of these clones (see Figure 9). The expression of mBCNG-1 appears to be restricted to the brain (Figure 9A), whereas mBCNG-2 (Figure 9B) and

mBCNG-3 (Figure 9C) are expressed in the brain as well as in the heart. Hybridization signals for mBCNG-3 are also detected in polyA$^+$ RNA from skeletal muscle and lung.

The distinct sequences and tissue distributions of these clones reveals that the BCNG clones represent a family of ion channel proteins, with characteristic voltage sensing and cyclic nucleotide binding motifs, that are predominantly located in heart and brain.

Results

The first fragment of mBCNG-2 (mBCNG2a [ATCC Accession No. 209825]) was cloned as a product of nested PCR reactions, designed to isolate 5' extensions of mBCNG-1 (Santoro et al., 1997). This fragment appeared to represent a distinct gene product from mBCNG-1 because of sequence differences in the overlapping region of the two PCR products. The differences were mostly third base codon substitutions. This fragment was used to screen a mouse brain λgt10 library; the N-terminal portion of a protein similar to, but distinct from mBCNG-1 was obtained (clone 11-λ1, designated mBCNG-2b [ATCC Accession No. 209826]). From the same λgt10 library screen, a weakly reacting plaque was also identified, which, upon subcloning and sequencing of the corresponding insert (clone 15-7, designated mBCNG-3a [ATCC Accession No. 209824]), was shown to represent a third distinct gene of the BCNG family (mBCNG-3). Seq.ID.No.: 5 represents mBCNG-2 DNA sequence while Seq.ID.No.: 6 represents mBCNG-2 amino acid sequence. Seq.ID.No.: 9 represents mBCNG-3 DNA sequence while Seq.ID.No.: 10 represents mBCNG-3 amino acid sequence.

A BLAST search in mouse and human EST databases revealed four EST clones that appear to be fragments of two mouse

BCNG genes (M41-EST, M28-EST) and two human BCNG genes (H57-EST, H61-EST).

The M41-EST sequence appeared to represent the 3' fragment of a BCNG-like gene, overlapping the cyclic nucleotide binding site. An oligonucleotide in this sequence (oligo 41REV [Seq.ID.No.: 24]) and an oligonucleotide in a conserved region of the 5' portion of the BCNG clones (oligo B123 [Seq.ID.No.: 23]), were synthesized and used to obtain RT-PCR products from mouse RNA. Sequencing of the RT-PCR product sections overlapping with the 5' end of the M41-EST clone and with the 3' end of mBCNG-2 cDNA (clone 11-λ1, designated mBCNG-2b [ATCC Accession No. 209826) established that M41-EST represents the 3' end region of mBCNG-2.

The M28-EST clone also appeared to contain a fragment of a BCNG-like gene, including the 3' end region of the cyclic nucleotide binding site. A degenerate oligonucleotide based on the M28 sequence was thus designed (oligo 28REV [Seq.ID.No.: 25]) and used together with the B123 oligonucleotide [Seq.ID.No.: 23]in an RT-PCR reaction on mouse RNA. Although the products obtained appear to represent extensions of mBCNG-3, as determined by the overlap with the 3' region of clone 15-7, sequencing reveled a difference in the overlapping region with the M28-EST clone. Thus, M28-EST represents yet another BCNG-like gene, which was designated mBCNG-4. Seq.ID.No.: 11 represents mBCNG-4 DNA sequence while Seq.ID.No.: 12 represents mBCNG-4 amino acid sequence. Complete sequencing of the M28-EST clone revealed that only the 3' end of the clone aligns with the BCNG sequences; the sequence 5' to position 632 is likely to represent an intron, and a stop codon is present at position 869 (see Figure 1).

Correspondence between these ESTs and the BCNG gene family is shown schematically in Figur s 7A-7B and is indicated in Table I. Clones were obtained from the IMAGE consortium. The clones were used in the following way.

TABLE I

EST CLONES IDENTIFIED BY HOMOLOGY TO mBCNG-1

Trivial Name	Probable Identity	GenBank Accession number	IMAGE consortium cDNA ID	Genome systems ID
M41-EST	3′ of mBCNG-2	AA023393	456380	
M28-EST	3′ of mBCNG-4	AA238712	693959	cd-22017
H61-EST	3′ of hBCNG-2	N72770	289005	
H57-EST	3′ of hBCNG-1	H45591	176364	

Table I lists the trivial names (designated herein), the probable correspondence between these ESTs and the BCNG genes, the GeneBank accession numbers and the clone identification numbers used by the I.M.A.G.E. consortium and Genome systems for these clones.

Predicted Amino Acid Sequence of the Conserved BCNG Channel Family. The deduced, integrated amino acid sequences obtained for the mBCNG-2 (Seq.ID.No.:6), mBCNG-3 (Seq.ID.No.:10) and mBCNG-4 (Seq.ID.No.: 12) encoded proteins are shown in Figures 8A-8B, and were aligned to the full length sequence of mBCNG-1 (Seq.ID.No.:2) (Santoro et al., 1997) (GenBank Accession No.: AF028737).

All of the identified sequences (except mBCNG-4) contain the motifs of a voltage-gated potassium channel (Jan and Jan, 1997). Thus, they appear to encode for channel subunits with an intracellular amino terminus, six putative transmembrane spanning domains (S1-S6), and a long cytoplasmic carboxy terminus. The S4 domain, which serves as the voltage sensor in other voltage-gated channels, contains 9 positively charged basic residues, more so than any other voltage-gated channel. In addition, the three clones contain a highly conserved pore-forming P region that links the S5 and S6 transmembrane segments. This P loop is homologous to the P regions of voltage-gated K channels and in particular contains the K channel signature sequence triplet, GYG, suggesting that the clones will encode a K selective ion channel (Heginbotham et al., 1994).

The long cytoplasmic tail of the BCNG proteins is predicted to contain a stretch of 120 amino acids that is homologous to the cyclic nucleotide binding (CNB) sites of cAMP- and cGMP-dependent protein kinases and the catabolite activating protein, CAP, a bacterial cAMP binding protein (Shabb and Corbin, 1992). The BCNG cyclic nucleotide-binding domains are most similar to the binding domains of the cyclic nucleotide-gated channels involved in visual and olfactory signal transduction (Zagotta and Siegelbaum, 1996). Although other members of the voltage-gated channel family have been reported to contain CNB sites, these putative sites lack many of the key conserved residues found in functional cyclic nucleotide-binding proteins (Tibbs et al., 1998). Strikingly, these key residues are conserved in the BCNG channel family, suggesting that these binding sites are likely to function.

Certain pacemaker channels can be regulated by PKA phosphorylation, such as the cardiac Purkinje fiber channel (Chang et al., 1991), whereas other pacemaker channels appear to be directly regulated by cAMP, such as the sino-atrial node channel (DiFrancesco and Tortora, 1991). It is interesting that mouse (and human) BCNG-1 and BCNG-2 contain a serine residue in their cytoplasmic carboxy terminus that lies within a consensus site for PKA phosphorylation (Figure 8B, arrow). mBCNG-4 does not contain this site, providing a potential explanation for the different modulatory properties of channels in different tissues. What is particularly striking about this potential phosphorylation site is that it lies within the C-helix of the cyclic nucleotide binding site, a region that forms a key part of the ligand binding pocket (Weber and Steitz, 1987). Studies on rod and olfactory cyclic nucleotide-gated channels previously showed that the C-helix plays an important role in ligand-selectivity and the efficacy of ligand-gating (Goulding et al., 1994; Varnum et al., 1995). Thus the phosphorylation of this serine residue might influence the efficacy with which cyclic nucleotides modulate the gating of certain pacemaker channels.

The mouse proteins are closely related to each other, having a similarity of 84-86% (Fig. 7C). Notably, mBCNG-2 and mBCNG-3 are more closely related to each other (89% similar) than either is to mBCNG-1. As far as a limited alignment could show (see legend to Figure 7), mBCNG-4 appears to be the most distantly related protein in the group.

Cloning of Two Human BCNG Genes. The high degree of similarity between H57-EST and mBCNG-1 suggested that this EST likely represented the 3' end region of the

human homolog of mBCNG-1 (designated hBCNG-1). Based on this assumption, PCR oligonucleotide primers were synthesized in order to amplify hBCNG-1. (See Figure 7). One primer consisted of a sequence in the 5' end of the mouse BCNG clones (oligo MB1-3 [Seq.ID.No.: 26]) and the second primer was based on a sequence in the H57-EST (oligo H57.C [Seq.ID.No.: 27]). (See Figure 7). A single, strong RT-PCR product of the predicted length was obtained using human brain polyA+ RNA. No band was obtained from human heart polyA+ RNA. Upon completion of the sequencing of the original EST clone and of the RT-PCR product, 2247 bp of the hBCNG-1 sequence was obtained (Seq.ID.No.: 3)(see Figure 7). The predicted amino acid sequence of the encoded hBCNG-1 protein (Seq.ID.No.: 4) is shown in Figure 8. Remarkably, the 308 amino acid-long core region of the hBCNG-1 protein, extending from the S1 through the S6 transmembrane segments, is 100% similar to mBCNG-1.

The H61-EST sequence showed marked sequence similarity to mBCNG-2 and could, in fact, encode the human homolog of the mBCNG-2 protein. Accordingly, a sequence within the H61-EST clone was used to probe a human brain λgt10 cDNA library. (See Figure 7). Combining the sequences of the H61-EST clone and of the λgt10 clones, 1792 bp of the hBCNG-2 sequence was obtained (Seq.ID.No.: 7)(See Figure 7). The predicted amino acid sequence of the encoded hBCNG-2 protein is shown in Seq.ID.No.:8. (See Figure 8). The 308 amino acid-long core region of the hBCNG-2 protein is 98% similar to mBCNG-2 (Fig. 7C).

Tissue Distribution of BCNG mRNA Expression. Northern blot analysis showed individual patterns of tissue distribution for each of the identified clones and a high correspondence in the transcript and localization

patterns between homologous mouse and human clones (Figs. 9 and 10). While the expression of mBCNG-1 appears to be restricted to the brain (Fig. 9, and Santoro et al. 1997), mBCNG-2 and mBCNG-3 are expressed in the brain as well as in the heart (Fig. 9). Hybridization signals for mBCNG-3 are also detected in polyA+ RNA from skeletal muscle and lung. A distinct pattern of tissue distribution is revealed for mBCNG-4, which appears to be mainly expressed in the liver, but is also present in brain, lung and kidney (Fig. 9D).

The homologous mouse and human BCNG genes are likely to be functionally similar since they exhibit very similar patterns of tissue expression as revealed by the Northern blot analysis. Figure 10 shows that a probe designed within the hBCNG-1 sequence recognized four transcripts in human brain polyA+ RNA. This pattern is very similar to that seen in the Northern blot of mBCNG-1 (Fig. 9 and Santoro et al. 1997). Weak hybridization signals are also detected for hBCNG-1 in human muscle and pancreas. Northern blot analysis using a probe based on the hBCNG-2 sequence showed an expression pattern which is highly consistent with the expression pattern of mBCNG-2 (Fig. 10; compare with Fig. 9). An abundant 3.4 kb transcript is detected in the brain and the same transcript is also present in the heart.

The analysis of the distribution of mBCNG-1 within the mouse brain (Santoro et al. 1997) revealed that the highest expression of mBCNG-1 occurs in the cortex, hippocampus and cerebellum. Moreover, the mBCNG-1 protein is specifically localized to the apical dendrites of pyramidal neurons as well as to the axon terminals of basket cells within these regions. (See, Example 1). Northern blot analysis of the hBCNG-1 mRNA distribution

within different brain regions also showed a differential expression of the gene, with the highest levels present in cortical structures (hippocampus and amygdala; Fig. 10). hBCNG-2 shows a more uniform level of high expression in all brain structures, suggesting a more ubiquitous role. In particular, the strong hybridization signal in corpus callosum-derived RNA may indicate expression of hBCNG-2 within glial cells.

EXPERIMENTAL PROCEDURES

Library screening and RT-PCR cloning. Standard manipulations of Escherichia coli, lambda phage and nucleic acids, including recombinant DNA procedures, were performed essentially as described (Sambrook et al. 1989).

Cloning of mBCNG-2. From the nested PCR reactions performed on the pJG4-5 library (see Example 1, Full-length cloning of mBCNG-1) an amplification product was isolated, that had a sequence similar but not identical to the expected mBCNG-1 sequence. It was thus inferred that it represented a different gene, closely related to mBCNG-1, which was designated mBCNG-2. The identified fragment ("dA") encoded amino acids 234-430 from the mBCNG-2 sequence (numbering according to mBCNG-1, see Figure 8)

Next performed was a series of RT-PCR reactions on polyA$^+$ RNA derived from mouse brain and heart, using oligos: 5'-TGGGAAGAGATATTCCACATGACC-3' (Seq. ID. No.: 19) (7.SEQ1, corresponding to amino acids 270-277 of the mBCNG-1 sequence; see Figure 8) as an upstream primer, and oligo d5.RL (Seq. ID. No.: 13) as a downstream primer. A 600 bp product was obtained from heart polyA$^+$ RNA, subcloned, sequenced and shown to be identical to mBCNG-2. PCR

cycling: 1x (2 minutes, 94°C); 25x (50 seconds, 94°C; 40 seconds, 52°C; 1.5 minute, 72°C); 1x (10 minutes, 72°C).

The Clontech Mouse Brain λgt10 library was screened at high stringency (see Example 1, Full-length cloning of mBCNG-1), with a PCR probe derived from the mBCNG-2 sequence (probe "dA") using oligos: 5'-TACGACCTGGCAAGTGCAGTGATGCGC-3' (Seq. ID. No.: 20) (ASEQ2, corresponding to amino acids 278-286 of the mBCNG-2 sequence, numbering according to mBCNG-1; see Figure 8) as an upstream primer, and 5'-AGTTCACAATCTCCTCACGCAGTGGCCC-3' (Seq. ID. No.: 21) (HRL.2, corresponding to amino acids 444-452 of the mBCNG-2 sequence, numbering according to mBCNG-1; see Figure 8) as a downstream primer.

Positively reacting clones were further screened by PCR, using oligonucleotide: 5'-CTGGTGGATATATCGGATGAGCCG-3' (Seq. ID. No.: 22) (BE-ASE, corresponding to amino acids 262-269 of the mBCNG-2 sequence, numbering according to mBCNG-1; see Figure 8) as a downstream primer and either of the two lambda derived oligonucleotides (15'.N2 (Seq. ID. No.: 17) or 13'.N2 (Seq. ID. No.: 18)) as an upstream primer (see Example 1, Full length cloning of mBCNG-1). The clones yielding the longest extension products were subcloned and sequenced, thus obtaining the N-terminal part of the mBCNG-2 sequence up to amino acids 304 (numbering according to mBCNG-1; see Figure 8).

After obtaining the sequence for EST-M41, a further round of RT-PCR reactions was performed both on mouse brain and heart polyA$^+$ RNA, using oligonucleotides: 5'-CAGTGGGAAGAGATTTTCCACATGACC-3' (Seq. ID. No.: 23) (B123, corresponding to amino acids 269-277 of the BCNG sequences, numbering according to mBCNG-1; see Figure 8)

as an upstream primer, and 5'-GATCATGCTGAACCTTGTGCAGCAAG-3' (Seq. ID. No.: 24) (41REV, corresponding to amino acids 590-598 of the mBCNG-2 sequence, numbering according to mBCNG-1; see Figure 8) as a downstream primer. Extension products of the expected length were obtained from both RNA preparations, subcloned and sequenced, linking the λgt10 derived 5′ fragment and the EST derived 3′ fragment of mBCNG-2.

PCR cycling was performed as follows: 1x (2 minutes, 94°C); 25x (45 seconds, 94°C, 30 seconds, 55°C); 2 minutes, 72°C);
1x (10 min, 72°C).

Cloning of mBCNG-3. From the λgt10 library screen for mBCNG-2 (see above) one positively reacting clone was obtained (#15) which appeared to give a consistently weaker hybridization signal. This insert was amplified with oligonucleotides 15.N2 (Seq. ID. No.: 17) and 13.N2 (Seq. ID. No.: 18), subcloned, sequenced and shown to represent a third BCNG-related sequence, different both from mBCNG-1 and mBCNG-2, which was called mBCNG-3. The identified fragment encoded the N-terminal part of the mBCNG-3 sequence up to amino acid 319 (numbering according to mBCNG-1; see Figure 8).

After obtaining the sequence for EST-M28, an RT-PCR was performed both on mouse brain and heart polyA$^+$ RNA using oligonucleotide B123 as an upstream primer, and degenerate oligonucleotide: 5'-CACCKCRTTGAAGTGGTCCACGCT-3' (Seq. ID. No.: 25) (28REV, corresponding to amino acids 554-561 of the BCNG sequences, numbering according to mBCNG-1; see Figure 8) as a downstream primer. Extension products of the expected length were obtained from both RNA preparations, subcloned and sequenced. Both

represented extension of the mBCNG-3 sequence, as determined by an overlap with the known 3' end of the λgt10 #15 clone. PCR cycling was performed at: 1x (2 minutes, 94°C); 25x (45 seconds, 94°C, 30 seconds, 55°C, 2 minutes, 72°C); 1x (10 minutes, 72 °C).

Cloning of hBCNG-1. After obtaining the sequence for EST-H57, an RT-PCR reaction was performed on human brain polyA$^+$ RNA, using oligonucleotides: 5'-ATGTTCGGSAGCCAGAAGGCGGTGGAG-3' (Seq. ID. No.: 26) (MB1-3, corresponding to amino acids 102-110 of the BCNG sequences, numbering according to mBCNG-1; see Figure 8) as an upstream primer, and 5'-CAGCTCGAACACTGGCAGTACGAC-3' (Seq. ID. No.: 27) (H57.C, corresponding to amino acids 537-544 of the hBCNG-1 sequence, numbering according to mBCNG-1; see Figure 8) as a downstream primer. A single extension product of the expected length was obtained, subcloned, sequenced, and shown to represent the 5' extension of the hBCNG-1 clone.

PCR was performed as follows: 1x (2 minutes, 94 °C); 25x (45 seconds, 94°C, 20 seconds, 58°C, 3 minutes, 72°C); 1x (10 minute, 72°C).

Cloning of hBCNG-2. After obtaining the sequence for EST-H61, a PCR probe was made using oligonucleotides: 5'AACTTCAACTGCCGGAAGCTGGTG3' (Seq. ID. No.: 28) (H61.A, corresponding to amino acids 452-459 of the hBCNG-2 sequence, numbering according to mBCNG-1; see Figure 8) as an upstream primer, and 5'GAAAAAGCCCACGCGCTGACCCAG3' (Seq. ID. No.: 29)(H61.F, corresponding to aa 627-634 of the hBCNG-2 sequence, numbering according to mBCNG-1; see Figure 8) as a downstream primer on the EST-H61 DNA. This fragment was used to screen a Human Brain Hippocampus cDNA library in λgt10 (CLONTECH, cat. no. HL

3023a), in high stringency conditions (see above). Positively reacting clones were further screened by PCR, using oligonucleotide:
5'CACCAGCTTCCGGCAGTTGAAGTTG3' (Seq. ID. No.: 30)(H61.C, corresponding to amino acids 452-459 of the hBCNG-2 sequence, numbering according to mBCNG-1; see Figure 8) as a downstream primer and either of oligonucleotides 15'.N2 (Seq. ID. No.: 17) or 13'.N2 (Seq. ID. No.: 18) as an upstream primer. The clones yielding the longest amplification products were subcloned and sequenced, thus obtaining the N-terminal region of the hBCNG-2 sequence up to aa 587 (numbering according to mBCNG-1; see Figure 8).

Northern blots. For mouse gene expression studies, a Mouse Multiple Tissue Northern Blot (CLONTECH, cat. no. 7762-1) was probed with the following PCR products: For mBCNG-1, probe "q0", obtained using oligos q0.5' (5'GCGAATTCAAACCCAACTCCGCGTCCAA3') (Seq. ID. No.: 31) and q0.3' (5'CCTGAATTCACTGTACGGATGGAT3') (Seq. ID. No.: 32). Amplification product corresponding to aa 6-131 of the mBCNG-1 sequence (see Figure 7 and Figure 8). For mBCNG-2, probe "dA", obtained using oligos ASEQ2/HRL.2 (see above). For mBCNG-3, probe "15-7", obtained using oligos 15.N2/13.N2 (see above); amplification performed directly on lambda phage DNA (clone #15). For mBCNG-4, probe "M28" was obtained as a gel-purified EcoRI/BglII restriction fragment (400 bp) from the EST-M28 DNA. Fragment corresponding to amino acids 529-607 of the mBCNG-4 sequence (numbering according to mBCNG-1; see Figure 8), plus 180 nucleotides of the mBCNG-4 3'UTR (untranslated region; see Seq. ID. No.: 11).

For human gene expression studies, a Human Multiple Tissue Northern Blot (CLONTECH, cat. no. 7760-1) or Human

Brain Multiple Tissue Northern Blot (CLONETECH, cat. no. 7750-1) was probed with the following PCR products: For hBCNG-1, probe H57, obtained using oligos H57.A (5'GTCGTACTGCCAGTGTTCGAGCTG3')(Seq. ID. No.: 33) and H57.B (5'GGTCAGGTTGGTGTTGTGAAACGC3') (Seq. ID. No.: 34). Fragment corresponding to aa 537-800 of the hBCNG-1 sequence (numbering according to mBCNG-1; see Figure 8). For hBCNG-2, probe "H61", obtained using oligos H61.A (Seq. ID. No.: 28) and H61.F (Seq. ID. No.: 29)(see above).

Hybridizations were all performed in ExpressHyb solution for 1 hour, 68°C, as indicated in the manufacturer's Protocol Handbook. Washing was performed as follows: 10 minutes, room temperature in 2x SSC/0.1% SDS, followed by twice 30 minutes, 65°C in 0.2x SSC/0.1% SDS. Filters were stripped between subsequent hybridizations by boiling for 5 min in 0.5% SDS/H_2O.

Sequence alignments and EST database search. Alignments and distance calculations were all performed with MegAlign (DNASTAR) on the indicated peptide sequences.

The EST database search was performed with BLAST (NCBI), using the mBCNG-1 polypeptide sequence (amino acids 1-720, to avoid the glutamine repeat present in the C-terminal region of the protein) and the TBLASTN program.

EXAMPLE 3 Physiological and Pharmacological Significance of Mouse and Human BCNG Channel Genes.

Introduction

The unique structural features and the tissue distribution of the predicted proteins of the BCNG gene

family suggested that they encode the pacemaker current (variously called Ih, If or Iq) of the heart and brain. Alternatively, it was suggested that it might be a component of other - perhaps unidentified - ionic current(s) that are important in cardiac renal, hepatic and central nervous system function.

The unique structural features of the predicted BCNG proteins (the unusual ion conducting pore (P) domain, the highly conserved cyclic nucleotide binding (CNB) site and the highly conserved and highly charged S4 voltage sensor) indicated that they may be susceptible to multiple drug intervention strategies that target the pore, the cyclic nucleotide binding site and the voltage-dependent gating apparatus.

Analysis And Predicted Structure and General Features of the BCNG Proteins. The predicted amino acid sequence of the BCNG genes revealed that they are members of the voltage-gated ion channel superfamily. Specifically, the BCNG proteins show similarities to the superfamily of channels that includes the voltage-gated K+ channels (Pongs, et al., 1995) and the cyclic nucleotide-gated channels, non-selective cation channels that are permeable to Na, K^+ and Ca (Zagotta and Siegelbaum, 1996). As shown schematically in Figure 11, the BCNG proteins are predicted to have six transmembrane spanning α-helices with cytoplasmic N and C termini, a highly basic fourth transmembrane domain (S4) and pore (p) region. Each of these motifs are found in the members of the voltage-gated K^+ family. In addition, the BCNG proteins have a well conserved cyclic nucleotide binding site in the C-terminus. Although a homologous motif is found in some of the voltage-gated K^+ channels, the cyclic nucleotide binding sites in those channels are not well conserved and there is little evidence that the binding

sites are functional. Indeed, the cyclic nucleotide binding site of the BCNG channels is most homologous to the sites found in cyclic nucleotide gated channels which use the binding of cyclic nucleotides to drive their activation. Furthermore, while the P loops of the BCNG channels are homologous to those found in voltage activated K^+ channels and cyclic nucleotide gated channels, there are several non-conservative changes in the amino acid sequence that are likely to yield ion conduction properties that are unique to the BCNG channels. Thus, the BCNG channels appear distinct from all previously identified channels in a number of ways which suggests that they have distinct physiological and pharmacological properties. These similarities and dissimilarities in the sequences of the voltage-gated K^+ channels, cyclic nucleotide-gated channels and the BCNG channels and the predicted consequences for BCNG channel functional properties are discussed more extensively below.

Results

The Hydrophobic Core. The core of BCNG channels is predicted to have six transmembrane (α-helical sequences (S1-S6) and a pore forming P loop. This assignment is homologous to a single subunit of the tetrameric K^+ channels (and tetrameric cyclic nucleotide-gated channels) or a single repeat in the pseudo tetrameric Na and Ca channels. This homology suggests that the BCNG channels are members of the voltage-gated K^+ channel superfamily (which also includes the voltage-independent but structurally homologous cyclic nucleotide-gated channels). It is likely that BCNG channels are composed of four such polypeptides in a hetero or homomultimeric structure as is seen for the voltage-gated K^+ channels and the cyclic nucleotide-gated channels (Chen et al., 1993;

Bradley et al., 1994; Liman and Back, 1994; Lin et al., 1996). However, mBCNG-1 shows considerable divergence from all other known K^+ channel and cyclic nucleotide-gated channel sequences. As noted above, the highest homology in the hydrophobic core region is to mouse Eag (22% amino acid similarity) - a voltage-gated K^+ channel that has a degenerate and probably non-functional cyclic nucleotide binding site Warimke and Gancleky. Over this core region, mBCNG-1 shows 17% identity to the voltage independent cyclic nucleotide-gated channels.

In contrast, the proteins that are predicted to be encoded by the BCNG genes show high homology to each other (> 80%, see Examples 1 and 2). Indeed, mouse BCNG-1 and human BCNG-1 are identical over the core region. Similarly, mBCNG-2 and hBCNG-2 are 98% identical over the core region. Thus, the BCNG family of genes appears to constitute a new branch of the K^+ channel superfamily which could be regulated by cyclic nucleotide binding. The presence of a gene family with members showing such sequence conservation strongly suggests important biological function.

The S4 voltage-sensing domain. The presence of positively charged arginine and lysine residues at every third position in the fourth transmembrane helix is a signature sequence of voltage-dependent gating (Hille, 1992; Catterall, 1992, see Figure 12). In contrast, in the voltage-independent cyclic nucleotide-gated channels, the S4 is degenerate with some of the positively charged residues being replaced by negatively charged acidic amino acids or being out of the triad repeat frame. These changes have reduced the net positive charge in the S4 of the cyclic nucleotide-gated channels to 3-4. This

introduction of negatively charged residues may underlie the reason that the cyclic nucleotide-gated channels no longer respond to voltage. However, it is also possible that voltage-sensitivity may have been lost as a result of some other structural change in the cyclic nucleotide-gated channels and the divergence in S4 structure is simply a reflection of the loss of evolutionary pressure to retain the positive charges.

The S4 of BCNG channels are most closely related to the corresponding regions in the voltage-gated K^+ channels *Shaker* and *eag*, albeit poorly (mBCNG-1 is 30% homologous to the S4 of *Shaker* and *eag*). Despite an interruption by the inclusion of a serine in place of an arginine in the S4 of BCNG channels, the BCNG sequence contains more positively charged residues than any other member of the voltage-gated K^+ channel superfamily (see Figure 12). The S4 domain of BCNG-1 has up to nine positively charged residues (one group of five and one group of four separated by a serine in place of one other arginine), which again makes it more similar to voltage activated K^+ channels (*Sh* and *eag* families) than to cyclic nucleotide-gated channels. The retention of such a highly charged S4 strongly suggests that the gating of these channels are voltage-sensitive.

The cyclic nucleotide binding site. Cyclic nucleotides regulate the activity of a diverse family of proteins involved in cellular signaling. These include a transcription factor (the bacterial catabolite activating protein, CAP), the cAMP- and cGMP-dependent protein kinases (PKA and PKG) and the cyclic nucleotide-gated (CNG) ion channels involved in visual and olfactory signal transduction (Shabb and Corbin, 1992; Zagotta and Siegelbaum, 1996). Despite obvious divergence among the

effector domains of these proteins, the cyclic nucleotide binding sites appear to share a common architecture. Solution of the crystal structures of CAP (Weber and Steitz, 1987) and a recombinant bovine PKA R1α subunit (Su, et al., 1995) has demonstrated that their cyclic nucleotide binding sites are formed from an α helix (A helix), an eight stranded β-roll, and two more α-helices (B and C), with the C-helix forming the back of the binding pocket. Of the approximately 120 amino acids that comprise one of these cyclic nucleotide binding sites, six are invariant in all CAP, PKA, PKG and cyclic nucleotide gated channels. Thus, it has been suggested that the invariant residues play important - and conserved - roles in the folding and/or function of the CNB sites of these diverse proteins (Shabb and Corbin, 1992; Zagotta and Siegelbaum, 1996; Weber and Steitz, 1987; Su, et al., 1995; Kumar and Weber, 1992; Scott, et al., 1996). Indeed, the crystal structure of CAP (Weber and Steitz, 1987) and the regulatory subunit of recombinant bovine R1α (Su, et al., 1995) reveals that the glycines are at turns within the β-roll while the glutamate and the arginine form bonds with the ribose-phosphate of the nucleotide.

Interestingly, only three of these residues - two glycines and the arginine - appear to be conserved among the more distantly related voltage-gated channels which bear the CNB site motif but whose gating may NOT be modulated significantly by direct binding of cyclic nucleotide (KAT1 (Hoshi, 1995) and drosophila EAG (dEAG) (Bruggeman, et al., 1993) (see Figure 11).

Thus in the plant channel, KAT1, the first glycine is mutated to an asparagine and the alanine is changed to a threonine. In dEAG the glutamate in changed to an

aspartate. Furthermore, the alignment of dEAG to the highly conserved RXA sequence in β-7 is uncertain. Often, the SAA sequence within the dEAG β-7 is aligned with the RXA consensus sequence which suggests that the arginine is lost and replaced with a serine. RAL is aligned with the RXA consensus sequence which would indicate that the arginine is retained but the alanine is replaced with a leucine. Regardless of which alignment is considered, it is clear that the binding site sequence of KAT1, dEAG and related channels all show deviations from the consensus motif for a functional cyclic nucleotide binding site. In keeping with this structural divergence, there is only one report that any cloned EAG is being sensitive to direct cyclic nucleotide binding (Bruggermann et al., 1993). However, this result has not been confirmed and it is now thought to be an artifact. There is some evidence that the gating of the plant channel KAT1, may be weakly sensitive to cyclic nucleotide modulation (Hoshi, 1995).

Figure 13 shows a schematic representation of the cyclic nucleotide binding site of bRET1 showing the critical interactions between the binding site and the cyclic nucleotide.

Recent evidence has demonstrated that the third (or C) α-helix moves relative to the agonist upon channel activation, forming additional favorable contacts with the purine ring. Indeed, the selective activation of bRET1 by cGMP relative to cAMP is largely determined by a residue in the C α-helix, D604 (Varnum et al., 1995). This acidic residue is thought to form two hydrogen bonds with the hydrogens on a ring nitrogen and amino group of the cGMP purine ring. Unlike the cGMP selective bRET1 channel, cyclic nucleotide-gated channels that are

activated equally well by cAMP or cGMP (fOLF1, Goulding et al., 1992; Goulding et al., 1994) or which favor activation by cAMP (rOLF2 coexpressed with rOLF1, Liman & Buck, 1994; Bradley et al., 1994) do not have an acidic residue here, but rather, have polar or hydrophobic amino acids (see Varnum, et al., 1995). Neutralization of D604 results in a loss of the ability to form the favorable hydrogen bonds with cGMP and the loss of the unfavorable interaction with the lone pair of electrons on the purine ring of cAMP, thus accounting for the channels which bear a hydrophobic or polar residue at this position becoming non-selective between cAMP and cGMP or even selective for cAMP.

In the C-terminus of the BCNG proteins there is a sequence of approximately 120 amino acids that is homologous to these cyclic nucleotide binding sites. Strikingly, the six residues that have been shown to be totally conserved in all functional cyclic nucleotide binding sites are conserved in all of the BCNG proteins that identified. The cyclic nucleotide binding site of the BCNG channels are most similar to the functional site present in the voltage-independent cyclic nucleotide-gated channels (30%). When the cyclic nucleotide binding sites found in channel genes are compared to those in protein kinases, the BCNG channel sites are more similar (25% similarity to yeast cAMP-dependent protein kinases) than those of any other ion channel. These data strongly suggest that the BCNG genes encode proteins whose activity is modulated by direct binding of cyclic nucleotide. Furthermore, the BCNG channels all have an isoleucine residue in the position where D604 is found in the cGMP selective bRET1 channel. Thus, the BCNG are suggested to be cAMP selective.

The pore. Despite the functional divergence that has given rise to Na, Ca or K selective families and to the presence of channels within these families whose conductances vary by 1-2 orders of magnitude, the pores of all members of the voltage-gated superfamily are related (see Itillic 1992; For example see Figure 14). Much is known about the residues that contribute to the ion permeation properties of channels and this allows predictions about the permeation properties of the BCNG proteins (Mackinnon, 1991; Heginbothem et al., 1994).

Overall, the P region of mBCNG-1 is most closely related to the corresponding region in the K selective *Shaker* and *eag* channels, albeit poorly (30%). Based on the presence of a GYG motif in the P loop, the BCNG proteins would be expected to be K selective. However, the BCNG P loops contain substitutions in several positions that are otherwise highly conserved in other voltage activated K+ channels. These changes can be seen in Figure 14 (which shows an alignment of mBCNG-1 against channels from all the other major K channel families) and Figure 8 (which shows the alignment of all currently cloned BCNG sequences). The aspartate residue which follows the GYG triplet is replaced with alanine (position 352) in mouse and human BCNG-1 and by an arginine in the other BCNG channels identified so far. The serine/threonine residue 8 residues N-terminal from that position (residue 344 in mBCNG-1) is replaced with histidine in all of the BCNG sequences. 6 residues N-terminal from the aspartate a hydrophobic leucine residue is introduced in place of a polar residue. In addition, at position -12 from the aspartate, a site that is occupied by an aromatic residue in all of the other channels aligned in Figure 14, a lysine residue is introduced in all of the BCNG sequences (Figure 8 and Figure 14).

Although the amino acid substitutions seen in the P region of the BCNG subunits do not necessarily indicate that the channel will have lost its K selectivity (for example a lysine is present in the P region of the K selective Shaw channel, See Figure 14) the substantial deviations from the K channel consensus sequence suggest that the BCNG proteins may generate a family of channels that do not select well between Na and K - consistent with the hypothesis that the BCNG channels encode the non-selective Ih pacemaker current.

Discussion

Significance of the BCNG Structure. The presence of cyclic nucleotide binding sites on a number of K^+ channels that are found in both plant and animal phyla suggests that the fusion between an ancestral K^+ channel and an ancestral cyclic nucleotide binding site is likely to have occurred prior to the evolutionary separation between plants and animals (Warmke and Ganetzky, 1994). Indeed, the finding that many of these sites are degenerate and non-functional supports this interpretation. Divergence from this common ancestor would have led on one hand to Eag-related channels (EAG, ERG, ELK) (Warmke and Ganetsky, 1994) and plant inward rectifiers (AKT and KAT), which maintained more of the features of voltage activated K^+ channels, while showing a progressive deviation from the original cyclic nucleotide binding site sequence) and on the other hand to CNG-channels (which show a higher evolutionary constraint on the cyclic nucleotide binding site, while they have lost voltage activation and K^+ selectivity) (Anderson, et al., 1992; Sentenac, et al., 1992). The features of BCNG-1 suggest that it may have remained closer to the ancestral molecule that represents the evolutionary link between voltage-gated K^+ channels

and cyclic nucleotide-gated channels. This is supported by the observations that the cyclic nucleotide binding site of mBCNG-1 shows the closest homology to binding sites present in protein kinases, in particular in yeast cAMP-dependent protein kinases (25%) while the channel domains are most closely related to the voltage-dependent channel encoded by the *Shaker* gene that does not have a cyclic nucleotide binding site and thus, presumably arose before the gene fusion event. The cyclic nucleotide binding site of mBCNG-1 is most homologous to the site present in cyclic nucleotide-gated channels (30%) which again demonstrates that these probably arose from a common ancestor and in both, there was pressure to maintain the cyclic nucleotide binding site because it contributed to the function of the protein. Thus, BCNG-1 appears to constitute a new branch of the K^+ channel superfamily.

Physiological Significance. Ion channels are central components underlying the electrical activity of all excitable cells and serve important transport functions in non-excitable cells. Members of the novel BCNG family of ion channels are expressed in both brain and cardiac muscle as well as skeletal muscle, lung, liver, pancreas and kidney. From their amino acid sequence, members of the BCNG channel gene family are likely to have important, novel roles in the electrophysiological activity of the brain and the heart and other tissues. This view is based, first, on the finding that mRNA coding for BCNG channel protein is expressed in both heart and brain. Second, the deduced primary amino acid sequence of the BCNG channels indicate that they are members of the voltage-gated channel family but unlike most voltage-gated channels, the BCNG channels contain what appears to be a functional cyclic nucleotide-binding

domain in their carboxy terminus.

Northern blots of the four mouse BCNG channel genes show interesting differences in expression patterns (see Figure 3, Figure 9 and Figure 10). mBCNG-1 is selectively expressed in brain. Western blots confirm that mBCNG-1 is also highly expressed at the protein level, and that this expression is widespread throughout the mouse brain (see Figure 2). mBCNG-2 is expressed in brain and heart. mBCNG-3 is expressed in brain, heart, lung and skeletal muscle. mBCNG-4 is expressed in brain, liver and kidney. Thus, each gene, although highly similar at the amino acid level, shows a distinct pattern of expression, implying that each has a unique physiological function. This is borne out by the finding that the two human members of the BCNG family, hBCNG-1 and hBCNG-2, show similar patterns of expression to the mouse homologs. Thus, hBCNG-1 is selectively expressed in brain (with weaker hybridization in pancreas) whereas hBCNG-2 is expressed in brain and heart. Even within a particular organ system, the different genes show different patterns of expression. Thus, in the brain hBCNG-1 is more highly expressed in hippocampus and amygdala than in other brain regions. In contrast, hBCNG-2 is highly expressed in all brain regions.

Based on the BCNG amino acid sequence and tissue distribution, it was hypothesized that the channels encode a either a voltage-gated potassium channel that is activated by membrane depolarization and modulated by the direct binding of cyclic nucleotides, or the hyperpolarization-activated pacemaker channel that underlies spontaneous electrical activity in the heart (DiFrancesco and Torta, 1991) and in various regions of the brain (Steride et al., 1993). This latter hypothesis

is based on the finding that the pacemaker channels, similar to BCNG genes, are expressed in both brain and the heart. Moreover, the pacemaker channels are known to be non-selective cation channels that are gated by both voltage and the direct binding of cyclic nucleotides to a cytoplasmic site on the channel (DiFrancesco and Torta, 1991; Pedarzani and Storm, 1995 Larkman And Kelly, 1997, McCormick and Page, 1990). To date, there is no biochemical or molecular biological information as to the nature of the pacemaker channel. However, the similarity in tissue distribution and proposed gating mechanisms between the pacemaker channels and the BCNG channels suggested that the BCNG genes code for one or more subunits that comprise the pacemaker channels.

Pacemaker channels have been studied at the electrophysiological level in both cardiac tissue and central neurons. In both instances, the channels are activated when the cell membrane voltage is made more negative than -40 mV. These non-selective channels are permeable to both Na and K+ ions. However, at the negative membrane potential range over which these channels open, their main effect is to allow positively charged sodium to enter the cell from the extracellular environment, causing the cell membrane to become more positive. This eventually causes the membrane voltage to reach threshold and the cell fires an action potential. (See Figure 15). Cyclic AMP (cAMP) is known to shift the relation between membrane voltage and channel activation, causing the channels to turn on more rapidly when the membrane is depolarized. This increases the rate of the pacemaker depolarization, increasing the rate of spontaneous action potential firing. It is this effect that underlies the ability of epinephrine (adrenaline) to cause the heart to beat faster. The effects of cAMP on the pacemaker current

appear to occur through two separate molecular mechanisms. First, cAMP activates the enzyme, cAMP-dependent protein kinase (PKA), leading to an increase in levels of protein phosphorylation. Second, cAMP is thought to directly bind to a cytoplasmic region of the pacemaker channel, producing an effect similar to that seen with protein phosphorylation. Such direct actions of cAMP have been reported both in the heart and in brain (DiFrancesco and Torta, 1991; Pedarzani and Storm, 1995).

An alternative function for the BCNG channels, that they encode for a novel voltage-gated and cyclic nucleotide-gated potassium channel, is suggested by the amino acid region that is known to line the ion-conducting pore and hence determine the ionic selectivity of the channel. This S5-S6 loop contains a three amino acid motif, GYG, that is conserved in almost all voltage-gated K^+ channels Heginbotham et al., 1994. The BCNG channel S5-S6 loop shows amino acid similarity with that of other potassium channels, including the GYG motif. This suggests that the BCNG channels may be K^+ selective. However, there are a number of striking differences in the sequence between BCNG and other K^+ channels that may indicate that the BCNG channels are less K-selective compared to other K^+ channels, consistent with the view that the BCNG channels code for the non-selective cation pacemaker channels that are permeable to both Na and K^+.

The presence of mBCNG-1 in the dendrites of hippocampal pyramidal cells is particularly intriguing, as cAMP has been shown to be important for the establishment of some forms of long-term synaptic potentiation in these cells (Frey, et al., 1993; Boshakov, et al., 1997; Huang and

Kandel, 1994; Thomas, et al., 1996). The structural features of mBCNG-1 predict a K^+ conducting activity, directly modulated by cyclic nucleotide binding.

Interestingly, a current with similar characteristics has been described in the hippocampal pyramidal neurons of area CA1 (Warmke, et al., 1991) where mBCNG-1 is highly expressed. This current (IQ) is believed to contribute to the noradrenergic modulation of hippocampal activity, by regulating neuronal excitability in response to cAMP levels. BCNG-1 may participate in the formation of the channels responsible for this type of current.

Based on the widespread tissue distribution and likely important physiological role of the BCNG channels in electrical signaling, drugs that interact with these channels are of potential therapeutic use in a number of neurological, psychiatric and cardiac diseases as well as systemic diseases of tissues such as skeletal muscle, liver and kidney.

Neurological disease: Based on the high expression of these channels in the hippocampus and potential role in spontaneous pacemaker activity, they may be useful, novel targets for treatment of epilepsy. For example, by blocking these channels it may be possible to prevent or diminish the severity of seizures. In diseases associated with hippocampal neuronal loss, such as age-related memory deficit, stroke-induced memory loss, and Alzheimers' disease, a drug which enhanced pacemaker channel activity may be of therapeutic use by increasing neuronal activity in the hippocampus. As these channels are also expressed in the basal ganglia and striatum, they may be potential targets in Parkinson's and Huntington's disease. The BCNG channels are also highly

expressed in the thalamus, where pacemaker channels have been shown to be important in generating spontaneous action potentials important for arousal. Targeting of such channels may help treat attention deficit disorder.

Psychiatric disease: Given the high levels of expression of hBCNG-1 in the amygdala, these channels may be targets for drugs involving various affective disorders and anxiety. Their high expression in the limbic system suggests that they may also be of potential benefit in treatment of schizophrenia.

Cardiac disease: The expression of the BCNG-2 channels in the heart suggests that they may be useful targets for treatment of certain cardiac arrhythmias. Based on the hypothesis that these genes may encode pacemaker channels, the BCNG channels will be potential targets for treating both bradyarrhythmias through drugs that enhance pacemaker channel activity and certain tachyarrhythmias due to enhanced automaticity. Even if the BCNG channels are not the pacemaker channels, they are likely to play an important role in cardiac electrical activity, perhaps contributing to action potential repolarization, and thus would remain attractive targets for drug development.

A number of drugs, toxins and endogenous compounds are known to interact with various types of ion channels. These drugs have proved useful as local anesthetics and in the treatment of cardiac arrhythmias, hypertension, epilepsy and anxiety. These drugs fall into several classes including pore blockers, allosteric modulators, and competitive antagonists (see Table II). The BCNG channels present some unique features that make them very attractive drugs. First, there are both brain specific genes (BCNG-1) and genes that expressed in both brain

and heart (BCNG-2,3). Thus BCNG may yield drugs specific for brain or heart. Second, the pore region of the BCNG channels shows considerable divergence from that of other known potassium channels. Thus, yielding pore-blocking drugs that would selectively alter BCNG channels but spare other types of voltage-gated K^+ channels. Third, the cyclic nucleotide-binding site elucidate another important target with respect to the opening of the BCNG channels. By designing specific cyclic nucleotide analogs it should be possible to design either synthetic agonists, which will increase channel opening, or antagonists which will decrease channel opening. Most available drugs for ion channels decrease channel opening, relatively few increase channel opening. The ability to either increase or decrease the opening of BCNG channels offers much potential for therapeutically effective compounds. For example in bovine photoreceptor CNG channels, Rp-cGMPS is an antagonist of channel opening whereas Sp-cGMPS is an agonist (Kramer and Tibbs, 1996). Moreover, the amino acid sequence of the cyclic nucleotide binding site of the BCNG channels shows considerable divergence with cyclic nucleotide binding sites of protein kinases and the cyclic nucleotide-gated channels of olfactory and photoreceptor neurons. Thus it should be possible to design cyclic nucleotide analogs which specifically target the BCNG channels.

TABLE II

Selected Drugs and Toxins That Interact with Members of the Voltage-gated Ion Channel Family

Compound	Channel Targets	Site of action	Therapeutic Uses
Local Anesthetics (lidocaine, procaine, etc.)	Na	Pore (S6)	Local analgesia, arrhythmias
diphenylhydantoin	Na	Pore	Seizures
Tetrodotoxin	Na	Pore	
Saxitoxin	Na	Pore	
α, β-Scorpion toxin	Na	Activation and Inactivation gates	
Dihydropyridines	Ca (L-type)	Pore	arrhythmias, hypertension, angina
Verapamil	Ca (L-type)	Pore	
Diltiazem	Ca (L-type)	Pore	
w-conotoxin	Ca (N-type)	Pore	
w-agatoxin	Ca (P-type)	Pore	
Tetraethyl-ammonium	K	Pore	
4-aminopyridine	K	Pore	
charybdotoxin	K	Pore	
hanatoxin	K	activation Gate	
amiodarone	K	?	arrhythmias
1-cis-diltiazem	CNG	Pore	
Rp-cAMPS	CNG	Binding site antagonist	
Sp-cAMPS	CNG	Binding site agonist	

Hille, 1992; Catterall, 1992; Roden, 1996.

From their amino acid sequence, these channels are likely to have three important physiological properties that make them *a priori* attractive targets for drug development. First, their gating should be voltage-dependent and thus it should be sensitive to modulation of the voltage-gating mechanism. Second, they possess a cyclic nucleotide binding domain in their C-terminus and it is probable that their gating will be modulated by direct binding of cyclic nucleotides. Third, the unusual sequence of the pore forming domain of the BCNG channels should allow the ion conduction properties of the channel to be selectively targeted.

If, the gating of the channels involves both the voltage-sensor machinery and the cyclic nucleotide binding site it is likely that coordinated drug regimes such that two compounds with low efficacy and even low selectivity can combine to selectively target the BCNG channels. Thus one compound that alone would have weak pharmacological effects on many voltage-activated channels combined with one that has a similarly weak effect on the various cyclic nucleotide binding pockets could be applied together. As no class of molecules is currently known that functionally combines BOTH of these structural elements - with the anticipated exception of the BCNG channels - it is likely that such a regime would lead to a highly efficacious and selective targeting of channels containing the BCNG subunits. Selective intervention against BCNG sub-types should also be possible.

The regulation of these channels through drugs provides a unique opportunity for regulating electrical activity associated with diseases as diverse as epilepsy and cardiac arrhythmias. Moreover, the cyclic nucleotide binding domain of these channels provides a unique

pharmacological target that could be used to develop novel, specific, cyclic nucleotide agonists or antagonists to upregulate or downregulate channel function.

Drugs can modulate voltage-dependent gating - coupled with CNG to achieve selectivity.

Cell lines expressing mBCNG-1, mBCNG-2, mBCNG-3, mBCNG-4, hBCNG-1 and hBCNG-2 offer the promise of rapid screening for compounds that interact with the channels. To identify drugs that interact with the cyclic nucleotide binding domain, this region could be expressed selectively in bacteria and then purified. The purified protein fragment could then be used in standard ligand-binding assays to detect cyclic nucleotide analogs that bind with high affinity.

Functional effects of drugs on channel opening or ion permeation through the pore are tested using whole cell patch clamp of mammalian cell lines expressing the various BCNG genes. Where the BCNG channels resemble the CNG channels, they exhibit significant permeability to calcium. This permits a high throughput screen in which channel function is assessed by imaging intracellular calcium concentration. Drugs that increase channel opening also increase internal calcium.

EXAMPLE 4: Functional Expression of mBCNG-1 in Xenopus Oocytes Reveals a Hyperpolarization-activated Cation Current Similar to Native Pacemaker Current. [Identification of a gene encoding a hyperpolarization-activated "pacemaker" channel of brain]

Introduction

The generation of pacemaker activity in heart and brain is mediated by hyperpolarization-activated cation channels that are directly regulated by cyclic nucleotides. We previously cloned a novel member of the voltage-gated K channel family from mouse brain (mBCNG-1) that contained a carboxy-terminal cyclic nucleotide-binding domain (Santoro et al., 1997) and hence proposed it to be a candidate gene for pacemaker channels. Heterologous expression of mBCNG-1 demonstrates that it does indeed code for a channel with properties indistinguishable from pacemaker channels in brain and similar to those in heart. Three additional mouse genes and two human genes closely related to mBCNG-1 display unique patterns of mRNA expression in different tissues, including brain and heart, demonstrating that these channels constitute a widely-expressed gene family.

The electrical activity of both the heart and the brain depends on specialized cells which act as pacemakers, generating the rhythmic, spontaneous firing of action potentials which can control muscle activity, certain rhythmic autonomic functions, and particular behavioral states. In normal nerve or muscle cells, pacemaker activity is characterized by spontaneous firing of action potentials that is intrinsic to the cell and independent of synaptic input. Defects in pacemaker activity can lead to both inherited (Spellberg, 1971) and acquired (Bigger and Reiffel, 1979) cardiac arrhythmias and may also underlie various neurological diseases.

In many of these cases, the pacemaker activity is generated by a hyperpolarization-activated channel that is permeable to both sodium and potassium and is present in both heart (DiFrancesco, 1993) and brain (Pape, 1996). Such cation-permeable channels were initially described in cardiac sinoatrial node cells (Brown et al., 1979; Yanagihara and Irisawa, 1980; Brown and DiFrancesco, 1980; DiFrancesco, 1986), where they were termed I_f or I_h. They have since been described in cardiac Purkinje fibers (DiFrancesco, 1981), ventricular muscle (Yu et al., 1993), and both peripheral (Mayer and Westbrook, 1983) and central neurons (Halliwell and Adams, 1982; see Pape, 1996 for review), where they are referred to as I_h or I_q. In sinoatrial node cells of the heart, the best studied example, this pacemaker channel drives the rhythmic firing and beating of the atria and ventricles (Brown et al., 1979; Yanagihara and Irisawa, 1980; Brown and DiFrancesco, 1980; although see Irisawa et al., 1993). In fact, it is through the modulation of this pacemaker channel that acetylcholine and norepinephrine exert their classical actions on heart rhythm.

In the brain, the modulation of pacemaker channel activity in thalamic relay neurons is important for regulating arousal during the sleep-wake cycle (Pape and McCormick, 1989; McCormick and Bal, 1997). Pacemaker activity in brainstem nuclei is likely to contribute to respiratory rhythms (Johnson and Getting, 1991; Dekin, 1993). Finally pacemaker activity in higher cortical regions is thought to contribute to endogenous oscillations that may be important for synchronizing the activity of neuronal populations (Maccaferri and McBain, 1996; Strata et al., 1997), a synchronization that has been proposed to bind together the separate analyzed components of a perceptual representation (Singer and Gray, 1995). Although the role of this channel in pacemaker activity may be its best characterized action,

it also contributes to rebound excitation following hyperpolarizing responses in non-pacemaking cells (Fain et al., 1978; Attwell and Wilson, 1980; Wollmuth and Hille, 1992; Halliwell and Adams, 1982; Mayer and Westbrook, 1983) and may have additional functional roles.

One striking feature of the pacemaker channels is that their activity can be modulated by transmitters and hormones acting through the second messenger cAMP (Tsien, 1974; DiFrancesco and Tortorra, 1991). Elevation of cAMP levels shifts the voltage-dependence of pacemaker channel activation by 2-10 mV in the positive direction. As a result, the channels activate more rapidly and more completely upon repolarization to a fixed, negative potential. Indeed, it is the ability of cAMP to modulate the activation of the pacemaker current that is largely responsible for the increase in heart rate in response to β-adrenergic agonists (Brown et al., 1979) and the slowing of the heart rate during vaginal stimulation (DiFrancesco et al., 1989; Zaza et al., 1996). Intriguingly, this effect of cAMP appears to be mediated through its direct binding to the channel in both sinoatrial node cells (DiFrancesco and Tortora, 1991) and in neurons (Pedarzani and Storm, 1995; Ingram and Williams, 1996). By contrast, I_f is regulated by PKA-dependent protein phosphorylation in cardiac Purkinje cells (Chang et al., 1991).

Pacemaker activity is characterized by spontaneous firing of action potentials in a nerve or muscle cell that is intrinsic to the cell and independent of synaptic input. This spontaneous firing is generated by a slow, pacemaker depolarization that is thought to involve the turning on of the hyperpolarization-activated pacemaker channels (DiFrancesco, 1993). Such cation-permeable channels were initially described in cardiac sinoatrial node cells

(Brown et al., 1979; Yanagihara and Irisawa, 1980; Brown and DiFrancesco, 1980; DiFrancesco, 1986), where they were termed I_f or I_h. They have since been described in cardiac Purkinje fibers (DiFrancesco, 1981), ventricular muscle (Yu et al., 1993), and both peripheral (Mayer and Westbrook, 1983) and central neurons (Halliwell and Adams, 1982; see Pape, 1996 for review), where they are referred to as I_h or I_q.

The pacemaker channels, unlike most voltage-gated channels, are closed when the membrane is depolarized during an action potential and only open when the membrane repolarizes to negative voltages. The opening of these channels upon repolarization of the action potential permits an influx of positively charged sodium ions, contributing to the spontaneous pacemaker depolarization. If this depolarization is of sufficient amplitude it can then trigger a second action potential, leading to repetitive, rhythmic, electrical activity. Although there is much evidence that supports a role for these channels in pacemaking (DiFrancesco, 1993; 1995; Pape, 1996), their exact quantitative contribution remains controversial (Irisawa et al., 1993; Vassalle, 1995).

One striking feature of the pacemaker channels is that their activity can be modulated by transmitters and hormones acting through the second messenger cAMP (Tsien, 1974; DiFrancesco et al., 1986). Elevation of cAMP levels shifts the voltage-dependence of pacemaker channel activation by 5-10 mV in the positive direction. As a result, the channels activate more rapidly and more completely upon repolarization to a fixed, negative potential. Indeed, it is the ability of cAMP to speed up the activation of the pacemaker current that is largely

responsible for the increase in heart rate in response to β-adrenergic agonists (Brown et al., 1979) and the slowing of the heart rate during vagal stimulation, when ACh acts through muscarinic receptor stimulation to decrease cAMP levels (DiFrancesco et al., 1989; Zaza et al., 1996). Intriguingly, this effect of cAMP appears to be mediated through its direct binding to the channel in both sinoatrial node cells (DiFrancesco and Tortora, 1991; DiFrancesco and Mangoni, 1994; Bois et al., 1997) and in neurons (Pedarzani and Storm, 1995; Ingram and Williams, 1996). By contrast, I_f is regulated by PKA-dependent protein phosphorylation in cardiac Purkinje cells (Chang et al., 1991).

Despite the intense physiological characterization of pacemaker function and mechanisms, the molecular nature of the hyperpolarization-activated cation channel that is responsible for generating the pacemaker depolarization has not yet been identified. For several reasons, we suspected that one candidate gene for the pacemaker channel might be mBCNG-1, which was originally cloned from a mouse brain cDNA library based on its interaction with the SH3 domain of a neural specific isoform of Src (Santoro et al., 1997). First, the deduced amino acid sequence of mBCNG-1 (originally termed BCNG-1 by Santoro et al.) reveals it to be a member of the superfamily of voltage-gated K channels (Jan and Jan, 1997), but with an unusual pore. Second, the carboxy terminus has a conserved cyclic nucleotide-binding (CNB) domain that is homologous to CNB domains of protein kinases (Shabb and Corbin, 1992) and the cyclic nucleotide-gated channels (Zagotta and Siegelbaum, 1996). This suggests its gating may be directly regulated by cyclic nucleotides. Third, both mBCNG-1 mRNA and protein are widely expressed in brain.

mBCNG-1 was a candidate gene for the pacemaker channel (Santoro et al., 1997), which codes for a member of the superfamily of voltage-gated K channels (Jan and Jan, 1997). This channel was originally cloned from a mouse brain cDNA library based on its interaction with the SH3 domain of a neural specific isoform of Src (n-Src). The channel protein and mRNA are widely expressed in brain. The deduced amino acid sequence of mBCNG-1 (Seq.ID.No.:2) (originally termed BCNG-1 by Santoro et al.) contains a carboxy terminus, cyclic nucleotide-binding domain that is homologous to CNB domains of protein kinases (Shabb and Corbin, 1992) and the cyclic nucleotide-gated channels (Zagotta and Siegelbaum, 1996). Based on the fact that mBCNG-1 appeared to encode for a subunit of a voltage-gated channel that could be directly regulated by cyclic nucleotides and its widespread distribution in the brain, it was suggested that mBCNG-1 might code for a subunit of the hyperpolarization-activated cation current (Santoro et al., 1997).

Here we report the functional expression of mBCNG-1 in Xenopus oocytes. Patch clamp recordings clearly demonstrate that this gene encodes a hyperpolarization-activated cation channel that is identical in its four key properties to the endogenous pacemaker channel of brain and also bears considerable similarity to that of the heart. Moreover, we report partial cDNA clones coding for three additional members of the BCNG family from mouse brain (mBCNG-2,3,4). These three additional clones are also expressed in a variety of other tissues, including the heart. Because of the potential clinical importance of this gene family, we have also isolated and characterized two human clones that are highly conserved with and show similar expression patterns to mBCNG-1 and mBCNG-2. Thus, the BCNG channel genes may encode not only pacemaker channels of the brain but they may also

encode a family of channels that are widely expressed in a variety of tissues, including the heart.

RESULTS

The location and deduced amino acid sequence of mBCNG-1, a gene which encodes a novel member of the superfamily of voltage-gated K channels, suggested to us that it might encode the hyperpolarization-activated pacemaker-type channel present in brain. To test this idea directly, we synthesized mBCNG-1 cRNA, expressed it in Xenopus oocytes, and analyzed the functional properties of the expressed channels in cell-free membrane patches. The native brain pacemaker channel has four distinctive properties: 1) it is activated with slow kinetics by hyperpolarization, 2) it is a cation channel that selects weakly for K relative to Na; 3) it is blocked by external Cs but not by Ba, and 4) it is directly modulated by intracellular cAMP. Expression of mBCNG-1 generates channels with each of these four properties as is demonstrated below.

Functional expression of mBCNG-1 in Xenopus oocytes reveals a hyperpolarization-activated cation current similar to native neuronal pacemaker current.

Patches obtained from oocytes injected with mBCNG-1 cRNA display hyperpolarization-activated ionic currents that resemble those seen in native neuronal pacemakers (Figures 16A-F). These currents activate when the membrane is hyperpolarized from a holding potential of -40 mV to test potentials below -80 mV (Figures 16A, 16B). The inward currents turn on with a relatively slow time course at less negative potentials but their rate of activation speeds up with increasing levels of hyperpolarization (Figs. 16A, 16E, 16F). Upon return to the holding potential (-40 mV), the currents deactivate relatively quickly, generating a decaying, inward tail

current (Fig 16C).

Hyperpolarizing steps to -130 mV typically activate between -20 pA to -200 pA of current among the >50 different patches from which we have recorded (patches with less than 5 pA of current were frequently observed but could not be analyzed). Such currents were not observed in uninjected control oocytes or in oocytes injected with cRNA encoding the bovine rod photoreceptor CNG channel, a voltage-independent channel that is activated by cGMP. As the single channel conductance of I_f is around 1 ps (DiFrancesco, 1986), we estimate that these patches (~ 1-2 μm^2 membrane area) typically contain 50-1500 mBCNG-1 channels, indicating a robust level of expression. The absence of discernable single channel currents in patches that display low current densities (< 2 pA), is consistent with mBCNG-1 channels displaying the small single channel conductance of the native pacemaker channels.

Both the time course of activation upon hyperpolarization and time course of deactivation upon return to the holding potential show characteristic sigmoidal kinetics (Figs. 16C, 16E), similar to those reported for the I_f current in native cells (DiFrancesco, 1984). Following an initial lag, the time course of activation could be approximated by a single exponential function (Fig. 16E), the time constant of which decreases from 290 ± 37 ms at -105 mV (mean ± s.e.m., n=5) to 98 ± 14 ms (n=5) at -130 mV (Fig. 16F, Table III).

TABLE III

Biophysical Properties of mBCNG-1

	CONTROL				cAMP		2 mM Cs
	$V_{1/2}$(mV)	Slope (mV)	τ_{-130} (ms)	τ_{-105} (ms)	$\Delta V_{1/2}$(mV)	Slope (mV)	%inhibition
MEAN	-99.9	-5.96	97.8	287.4	1.8	-6.30	92.4
SEM	0.8	0.71	13.6	37.0	0.3	0.71	2.5
n	5	5	5	5	5	5	6

TABLE III. SUMMARY OF BIOPHYSICAL PROPERTIES OF mBCNG-1. Control. Average values for steady-state activation parameters: $V_{1/2}$ and slope, from Boltzmann equation fit to tail-current activation curves; mean time constants of activation for steps to -130 and -105 mV. cAMP. Mean effect of cAMP on $V_{1/2}$ and slope of steady-state activation curve. Shift in $V_{1/2}$ measured by averaging $V_{1/2}$ values before cAMP and after washout and subtracting this average from $V_{1/2}$ value in presence of cAMP (1, 30 or 3000 μM). Slope gives mean slope in presence of cAMP. 2 mM Cs. Mean percent block of mBCNG-1 current by Cs. Second and third lines show standard errors and number of experiments for each measurement.

The steady-state voltage-dependence of activation was determined by hyperpolarizing the membrane to various test voltages (Fig. 16C). The relative magnitude of the decaying inward tail current upon return to the holding potential of -40 mV provides a measure of the fractional activation of the current during the preceding hyperpolarization. The peak tail current amplitudes show a sigmoidal dependence on the test voltage (Fig. 16D).

They begin to activate at potentials negative to -80 mV and reach maximal activation with steps to between -110 and -120 mV. Fits of the Boltzmann equation to this relation (see Experimental Procedures) yield estimates of the voltage at which mBCNG-1 is half-maximally activated ($V_{1/2}$ = -99.9 ± 0.8 mV, n=5) as well as the slope of the relation between voltage and activation (e-fold change for -6.0 ± 0.7 mV, n=5).

The mBCNG-1 current is carried by K and Na

Native pacemaker channels are weakly selective for K over Na, exhibiting typical reversal potentials of around -30 mV under physiological gradients of Na and K. To demonstrate that the mBCNG-1 current is indeed mediated by cation-conducting channels, we measured the reversal potential of these currents under conditions of symmetrical Cl concentrations but asymmetric concentrations of K and Na that approximate their physiological gradients (Figs. 17A, 17C). We find that the reversal potential of the mBCNG-1 current occurs at -31.8± 1.6 mV (n=4), very close to the expected value (Figs. 17A-C). As the above measurements were obtained in the absence of added Ca, we next explored the possibility that these channels may be converted to Ca-selective channels in the presence of external Ca, similar to voltage-gated calcium channels (Hess and Tsien, 1983). However, we found that addition of 1 mM Ca to the external NaCl solution did not cause any positive shift in the reversal potential (-34.9 ± 3 mV, n=2), which would be expected for a Ca-selective channel.

The reversal potential that we determined for the mBCNG-1 current is also clearly distinct from the value of 0 mV expected for a current carried by chloride ions. This point is important as Xenopus oocytes contain endogenous hyperpolarization-activated Cl channels whose level of expression can change upon expression of cRNAs

(Tzounopolous et al., 1995). To further rule out a role for Cl, either as a charge carrier through mBCNG-1 channels or as a current that contaminates our measurements of mBCNG-1 currents, we replaced the internal Cl by aspartate, an anion which does not permeate most Cl channels. This is expected to shift the Cl equilibrium potential to -78 mV. Although Cl replacement altered the magnitude of the mBCNG-1 current (similar to that previously reported for the native I_f channels - Frace et al., 1992), it did not cause a negative shift in the reversal potential (Figs. 17B, 17C; there may be a small positive voltage shift that might be due to changes in liquid junction potential or K ion activity). It was thus concluded that mBCNG-1 does indeed code for a hyperpolarization-activated cation channel that is permeable to K and Na, similar to native I_f and $_hI$ currents. Based on the measured reversal potential and the Goldman-Hodgkin-Katz equation, the channel is 4-fold more permeable to K than to Na, similar to the ratio in native pacemaker channels.

The mBCNG-1 current is blocked by external Cs but not by external Ba

A characteristic feature of the pacemaker channel that allows it to be distinguished from several other types of hyperpolarization-activated channels is its sensitivity to block by relatively low concentrations of extracellular Cs (DiFrancesco, 1982; Noma et al., 1983). At the same time, the pacemaker channels are relatively insensitive to block by external Ba (DiFrancesco, 1982). We find that the mBCNG-1 channels are similar to native pacemaker channels in their sensitivity to external cations. Thus, the mBCNG-1 current is nearly completely blocked by 2mM Cs ions when applied to the extracellular surface of an outside--out patch (Figs. 18A-18B; mean % inhibition = 92.4 ± 2.5, n=6). Dose response curves for this effect show that the IC_{50} is around 200 μM with a

Hill coefficient of ~1 (Fig. 18C). In contrast to the blocking action of Cs, addition of 1 mM Ba to the external solution, which blocks inward rectifier K channels and hyperpolarization activated Cl channels, had little effect (Figs. 18A-B; mean percent inhibition = 0 ± 5 %, n=3). The fact that mBCNG-1 channels are largely blocked by Cs also indicates that our current measurements are not contaminated to any significant extent by endogenous oocyte Cl channels (Barish, 1983; Tzounopolous et al., 1995) or stretch-activated cation channels (Yang and Sachs, 1990), neither of which are blocked by external Cs.

mBCNG-1 channels are directly modulated by cAMP

Previous studies on native pacemaker channels have shown that direct application of cAMP and/or cGMP to cell-free inside-out patches can increase the size of the I_f current elicited by a submaximal hyperpolarization, due to a shift of the activation curve to more positive potentials (DiFrancesco and Tortora, 1991). We observed a qualitatively similar effect with the mBCNG-1 channels (Figs. 19A-C). Thus, in response to bath application of cAMP to the internal surface of an inside-out patch, there is a reversible increase in the magnitude of the inward current during a step to 100 mV. The increase in current is observed in response to 1 μM, 30 μM or 3 mM cAMP (Figs. 19A, 19B).

This effect of cAMP is due to a small, but reproducible, positive shift in the steady-state activation curve of these channels by 2 mV (Fig. 19C; mean = 1.8 ± 0.3 mV, n=5). Although this effect of cAMP is small, it was consistently observed in 5 out of 5 patches and found to be statistically significant ($P<0.001$; paired t-test). Moreover, in the four patches in which we could record full activation curves after washout of cAMP, the shift was shown to be reversible. The voltage shift is also

observed when the individual activation curves from the 5 separate experiments are averaged to obtain the mean activation curves in either the absence or presence of cAMP (Fig. 19C). Although the averaging procedure tends to obscure the effects of cAMP due to small variability in the control activation curves among different patches, the difference between the curves is still significant ($P<0.05$; two way ANOVA with one repeated measure, $F(3,6)=11.56$). Furthermore, the shift we observe here with cAMP is nearly identical to the shift observed for pacemaker channels in the hippocampus (Pedarzani and Storm, 1995), a region where mBCNG-1 is highly expressed (Santoro et al., 1997). As there was no ATP or GTP in the internal solutions, cAMP is likely to act by directly binding to the channels.

Based upon the above observations, we concluded that the hyperpolarization-activated current observed upon mBCNG-1 expression represents expression of a pacemaker channel with voltage-dependence, ionic selectivity, ionic blocking properties and second messenger modulation similar to the native brain pacemaker channels.

Identification of a family of BCNG genes

Since mBCNG-1 is expressed only in brain, we wondered whether other, related genes may be expressed in different tissues, including the heart. We have isolated partial cDNA clones for three additional mouse and two human genes encoding regions homologous to mBCNG-1. Partial cDNA clones representing two mouse genes (mBCNG-2 and mBCNG-3) were isolated while screening for full-length mBCNG-1 products, and a fourth mouse gene (mBCNG-4) as well as two human genes (hBCNG-1 and hBCNG-2) were identified following an EST database homology search, using the protein sequence of mBCNG-1 as a query. Further extensions of the identified cDNA clones were subsequently obtained by library screening or RT-PCR

cloning. A schematic representation of the mouse and human BCNG sequences identified so far is presented in Figures 7A-7B.

Predicted amino acid sequence of the conserved BCNG channel family

The deduced, integrated, partial sequences obtained so far for the mBCNG-2, mBCNG-3 and mBCNG-4 encoded proteins are shown in Figures 8A-8B, and are tentatively aligned to the previously reported full length sequence of mBCNG-1 (Santoro et al., 1997). All of the identified sequences (except mBCNG-4) contain the conserved motifs of a voltage-gated potassium channel (Jan and Jan, 1997), including the S1-S6 transmembrane segments, a charged S4 voltage-sensor, and a pore-lining P loop. In addition, all BCNG family members contain a conserved cyclic nucleotide-binding domain in their carboxy terminus. It is interesting that both mBCNG-1 and mBCNG-2 contain a serine residue in their cytoplasmic carboxy terminus that lies within a consensus site for PKA phosphorylation (Figures 7a-7B , arrow) whereas mBCNG-4 does not contain this site. The absence or presence of the PKA site on different channels may explain why the cardiac Purkinje fiber channel is regulated by PKA phosphorylation (Chang et al., 1991) whereas the sinoatrial node channel is directly regulated by cAMP (DiFrancesco and Tortora, 1991).

The three mouse proteins are closely related to each other, having a similarity of 84-86% over the hydrophobic core region (amino acids 111 through 419, numbered according to mBCNG-1). Notably, mBCNG-2 and mBCNG-3 are more closely related to each other (89% similar) than either is to mBCNG-1. As far as a limited alignment could show, mBCNG-4 appears to be the most distantly related protein in the group (amino acids 529 through 592), having a similarity of 79% to mBCNG-1. The 308

amino acid-long core region of the hBCNG-1 protein, extending from the S1 through the S6 transmembrane segments, is 100% identical to mBCNG-1, while the core region of the hBCNG-2 protein is 98% similar to mBCNG-2.

Tissue distribution of BCNG mRNA expression

Northern blot analysis showed individual patterns of tissue distribution for each of the identified clones and a high correspondence in the transcript and localization patterns between homologous mouse and human clones (Figs. 9A-9D and 10A-10D). The expression of mBCNG-1 appears to be largely restricted to the brain (Figs. 9A-D; probe "q1", see Fig. 7A-7B), as previously reported using a distinct amino terminus probe (probe "q0"; Santoro et al. 1997). In contrast, mBCNG-2 and mBCNG-3 are expressed in the brain as well as in the heart (Figs. 9A-D). Hybridization signals for mBCNG-3 are also detected in poly A+ RNA from skeletal muscle and lung. A distinct pattern of tissue distribution is revealed for mBCNG-4, which appears to be mainly expressed in the liver, but is also present in brain, lung and kidney (Figs. 9A-D).

The homologous mouse and human BCNG genes are likely to be functionally similar since they exhibit very similar patterns of tissue expression as revealed by Northern blot analysis. Figures 10A-D shows that a probe designed within the hBCNG-1 sequence recognized four transcripts in human brain polyA+ RNA, similar to that seen in the mBCNG-1 Northern blot (Fig. 9A-9D and Santoro et al. 1997). Weak hybridization signals are also detected for hBCNG-1 in human muscle and pancreas. Northern blot analysis using a probe based on the hBCNG-2 sequence showed an expression pattern which is highly consistent with the expression pattern of mBCNG-2 (Fig. 10A-D; compare with Figs. 9A-9D). An abundant 3.4 kb transcript is detected in the brain and the same transcript is also present in the heart.

The analysis of the distribution of mBCNG-1 within the mouse brain (Santoro et al. 1997) revealed that the highest expression of mBCNG-1 occurs in the cortex, hippocampus and cerebellum. Northern blot analysis of the hBCNG-1 mRNA distribution within different brain regions also showed a differential expression of the gene, with the highest levels present in cortical structures (hippocampus and amygdala; Figs. 10A-D). hBCNG-2 shows a more uniform level of high expression in all brain structures, suggesting a more ubiquitous role. In particular, the strong hybridization signal in corpus callosum-derived RNA may indicate expression of hBCNG-2 within glial cells.

If the BCNG-2 and BCNG-3 genes expressed in heart do indeed code for pacemaker channels, we would expect them to be expressed in the cardiac tissues in which pacemaker channel activity has been reported. Although the sinoatrial node is the primary pacemaking tissue of the heart, latent pacemaker activity is found in both atrial (Thuringer et al., 1992; Zhou et al., 1992) and ventricular (Yu et al., 1993; Robinson et al., 1997) muscle. However, the voltage-dependence of activation of the channels in the regions outside of the node is normally shifted to very hyperpolarized, non-physiological potentials. In accord with this observation of a widespread occurrence of pacemaker channels throughout the heart, we find that primers which amplify both mBCNG-2 and mBCNG-3, but not mBCNG-1, generate RT-PCR products from ventricular, atrial and sinoatrial node mRNA (Figure 20A). To establish the relative expression of mBCNG-2 and mBCNG-3 within each of these regions of the heart, we performed a Southern blot analysis on the RT-PCR products. Hybridization with probes that specifically recognize mBCNG-2 or mBCNG-3 demonstrate that the predominant species within the RT-PCR product, and hence the analyzed cardiac regions, is

mBCNG-2 (Figs. 20B, C).

DISCUSSION

Identification of a pacemaker channel gene for brain

The distinct sequences and tissue distributions of the identified BCNG genes reveals that the BCNG products represent a family of ion channel proteins, with characteristic motifs for voltage-sensing and cyclic nucleotide-binding. These genes are predominantly located in brain and in heart. When expressed in Xenopus oocytes, the mBCNG-1 channel gives rise to a hyperpolarization-activated cation channel whose properties closely correspond to those of the pacemaker current in the heart (I_f). Although we have no direct evidence that the members of the BCNG channel family expressed in heart code for cardiac pacemaker channels, the tissue distribution and sequence similarity of these partial cDNA clones with full-length mBCNG-1 is suggestive that they may well code for the cardiac channel.

Members of the voltage-gated K channel family are generally tetramers, composed of four pore-forming subunits (MacKinnon, 1991). Although mBCNG-1 cRNA leads to expression of functional channels on its own, the existence of multiple BCNG genes suggests that the native channels may be heteromultimers. In addition, the BCNG channel subunits may associate with auxiliary, non-pore-forming β subunits that modify the function of the channel. It is possible that mBCNG-1 may be more potently modulated by cAMP when it combines with additional subunits. Alternatively, there may be inherent differences in the efficacy with which cAMP modulates different BCNG family members. In support of this latter possibility, the cAMP-dependent shift of Ih in hippocampal neurons, where BCNG-1 is prominently

expressed (Figs. 10A-D, and Santoro et al., 1997), is only 2-3 mV (Pedarzani and Storm, 1995), almost identical to the shift observed here. This contrasts with the 10 mV shift reported for I_f in sino-atrial node (DiFrancesco and Tortora, 1991) and the 4-6 mV shift of I_h in sensory neurons (Ingram and Williams, 1966). The relatively rapid activation kinetics of mBCNG-1 also make it more similar to the rapidly activated hippocampal channel (Halliwell and Adams, 1982; Maccaferri et al., 1993; Pedarzani and Storm, 1995) than to the more slowly activating cardiac channels. It is an intriguing possibility that differences in cAMP efficacy may be related to the phosphorylation state of the serine residue in the PKA consensus site within the cyclic nucleotide binding domain of the BCNG-1 and BCNG-2 subunits.

Two important differences from classic voltage-gated channels endow the mBCNG-1 channel with its characteristic properties

Despite the sequence similarity between the BCNG channels and other voltage-gated K channels, there are two important functional differences. First, most voltage-gated K channels are usually activated by depolarization. The opposite voltage-dependent polarity of the mBCNG-1 channels occurs despite the presence of a highly charged, basic S4 voltage-sensing domain. Second, whereas most members of the K channel family are at least 100-fold selective for K ions over Na ions, the mBCNG-1 channels, like the native pacemaker channels, are only four fold selective for potassium versus sodium. The BCNG channels do, however, contain a P region that is similar to P regions of other K selective channels. We discuss possible mechanisms for these differences below.

How can the BCNG channels activate upon membrane hyperpolarization rather than depolarization, despite the

presence of an S4 segment? A similar reversed polarity of voltage-dependent activation has been reported for the S4-containing KAT1 voltage-gated potassium channel of plants (Schachtman et al., 1994). One relatively simple mechanism that can explain the voltage-gating properties of KAT1, and mBCNG-1 channels is provided by the study of Miller and Aldrich (1996) on Shaker K channels, which normally activate rapidly and then inactivate rapidly upon depolarization. These authors showed that S4 point mutations that shift the activation gating reaction to very negative potentials (well below the resting potential) transformed the Shaker K channels into hyperpolarization-activated channels. At voltages near the resting potential, even though the activation gates of the mutant channels are in the open configuration, the channels are closed due to the inactivation reaction. Moderate hyperpolarizations, that are not sufficiently negative to shut the activation gates, open the channels by causing inactivation gates to open. The opening of the BCNG channels upon hyperpolarization could reflect a similar removal of inactivation.

The lower K selectivity of the mBCNG-1 channels could reflect the presence of several non-conservative changes at key positions in the P region. Thus, although the channels do contain the GYG motif that forms the major part of the K channel selectivity sequence, a conserved threonine residue two residues N terminal to the GYG triplet is changed to a cysteine in the BCNG channels. This conserved threonine has been shown to form part of the K selectivity filter in the X-ray crystal structure of the bacterial kcsa channel (Doyle et al., 1998). Moreover, a highly conserved aspartate (immediately C terminal to the GYG triplet) is either an alanine or arginine in the BCNG clones.

The role of the pacemaker current in disease

Might changes in BCNG channel function due to posttranslational modification, changes in gene expression, or genetic mutations underlie inherited or acquired neurological disorders or diseases of automaticity? With our identification that the BCNG genes code for CNS and, perhaps, cardiac pacemaker channels, it should now be possible to determine whether certain familial sinus rhythm diseases (Spellberg, 1971) are due to primary defects in the pacemaker channel. Defects in pacemaker channel function could also contribute to acquired diseases of the heart, such as sick sinus syndrome associated with atrial fibrillation, sinus tachycardias and bradycardias (Bigger and Reiffel, 1979), and ventricular arrhythmias associated with heart failure (Cerbai et al., 1994; 1997).

The existence of multiple genes coding for regionally specific channels offers the intriguing possibility of developing therapeutic agents that would specifically target, for example, cardiac rather than brain pacemaker channels. Conversely, the importance of pacemaker activity in the brain for arousal and perhaps perceptual awareness might make these brain channels interesting targets for pharmacological manipulation. Finally, controversies as to the precise role of the pacemaker channels in the electrical activity of both the brain and heart should now be amenable to the powerful approaches of mouse genetics.

Experimental Procedures

Library screening and RT-PCR cloning

Standard manipulations of Escherichia coli, lambda phage and nucleic acids, including recombinant DNA procedures, were performed essentially as described (Sambrook et al. 1989).

The first fragment of mBCNG-2 cDNA was cloned as a product of PCR reactions designed to isolate full length mBCNG-1 cDNA (Santoro et al., 1997). This fragment corresponded to 234-430 of the mBCNG-2 sequence (numbering here and throughout according to mBCNG-1, see Figures 8A-8B) and was used to screen a mouse brain λgt10 library (CLONTECH® ML 3000a) at high stringency, yielding the N-terminal part of mBCNG-2 (clone 11-λ1). From the same λgt10 library screen, a weakly reacting plaque was also identified (clone 15-7), which was subsequently shown to represent a third distinct gene (mBCNG-3).

A BLAST search in mouse and human EST databases revealed four EST clones that appeared to be fragments of two mouse BCNG genes (M41-EST, gb AA023393; M28-EST, gb AA238712) and two human BCNG genes (H57-EST, gb H45591; H61-EST, gb N72770). Oligonucleotides were designed within these sequences and used together with oligonucleotides designed in conserved regions of the BCNG clones to obtain RT-PCR products from mouse or human polyA+ RNA (see below). RT-PCR products were sequenced and the overlapping regions compared, to establish the correspondence between ESTs and known BCNG clones (see Figure 5). The M28-EST clone appeared to represent a fourth and distinct gene from the previously identified BCNG cDNAs. Complete sequencing of the M28-EST clone revealed that only the 3' end of the clone aligns with the BCNG sequences; the sequence 5' to position 632 is likely to represent an intron, and a stop codon is present at position 869.

A human brain λgt10 library (CLONTECH®, HL 3022a) was screened with a fragment derived from H61-EST clone (probe "H61"); see below, and Figures 7A-C), yielding the N-terminal region of the hBCNG-2 sequence up to amino acid at position number 587.

RT-PCR reactions were performed (25 times for 45 sec at 94 °C, 30 sec at 55 °C, and 2 min at 72 °C) on polyA+ RNA preparations from mouse brain and mouse heart (CLONTECH®, 6616-1 and 6611-1), using the SuperScript Preamplification System (GIBCO-BRL®) with the following oligonucleotides:

B123 5'CAGTGGGAAGAGATTTTCCACATGACC3' (Seq I.D. No. 23) (corresponding to aa 269-277) and 41REV 5'GATCATGCTGAACCTTGTGCAGCAAG3' (Seq. I.D. No. 24) (corresponding to aa 590-598) or 28REV 5'CACCKCRTTGAAGTGGTCCACGCT3' (Seq I.D. No. 25) (corresponding to aa 554-561).

For human genes, reactions were performed (25 times for 45 sec at 94 °C, 20 sec at 58 °C, and 3 min at 72 °C) on polyA+ RNA from human brain and human heart (CLONTECH®, 6516-1 and 6533-1) using the following oligonucleotides:

MB1-3 5'ATGTTCGGSAGCCAGAAGGCGGTGGAG3' (Seq I.D. No. 26) (corresponding to aa 102-110) and H57.C 5'CAGCTCGAACACTGGCAGTACGAC3' (Seq I.D. No. 27) (corresponding to aa 537-544).

For heart region localization of BCNG-2/3 transcripts, reactions were performed on polyA+ RNA isolated from ventricles, atria or sinoatrial nodes of rabbit heart, using oligonucleotides:

BCNG-2/F2 5'GAGCAGGAGCGCGTCAAGTCGGCG3' (Seq I.D. No. 35) (corresponding to aa 112-119) and BCNG-2/R2 5'GAAGATGTAGTCCACGGGGATGGA3' (Seq I.D. No. 36) (corresponding to aa 218-225).

To determine the specificity of recognition among different family members, the same oligonucleotides were

used on plasmid DNAs that encoded mBCNG-1, 2 or 3 cDNAs (see Legend to Fig. 9A). These plasmids were labeled by random priming (STRATAGENE®) and used as the probes for the Southern blot analysis.

Northern Blots

For mouse gene expression studies, a mouse multiple tissue Northern blot (CLONTECH®, 7762-1) was probed with PCR products representing the following regions of the mBCNG clones (see schematic representation in Figs. 7A-7B and amino acid numbering in Figs. 8A-8B):

mBCNG-1: probe "q1" (corresponding to aa 594-720; Santoro et al., 1997);
mBCNG-2: probe "dA" (corresponding to aa 234-430).
mBCNG-3: probe "15-7" (corresponding to the mBCNG-3 sequence from start up to position 319).
mBCNG-4: probe "M28" (corresponding to aa 529-607 of the mBCNG-4 sequence plus 180 nt of the mBCNG-4 3' UTR; this probe was obtained as a gel-purified EcoRI/BglII restriction fragment, 400 bp, from the EST-M28 DNA).

For human gene expression studies, a human multiple tissue Northern blot (CLONTECH®, 7760-1) or human brain multiple tissue Northern blot (CLONTECH, 7750-1) was probed with the following PCR products:

hBCNG-1: probe "H57" (corresponding to aa 537-800).
hBCNG-2: probe "H61" (corresponding to aa 452-634).
Hybridizations were all performed in EXPRESSHYB® solution (CLONTECH®) for 1 hr at 68 °C, as indicated in the manufacturer's Protocol Handbook. Blots were washed for 10 min at room temperature in 2x SSC/0.1% SDS, followed by two washes for 30 min at 65 °C in 0.2x SSC/0.1% SDS. Filters were stripped between subsequent hybridizations by boiling for 5 min in 0.5% SDS/H2O.

Electrophysiological recordings

mBCNG-1 was subcloned into the pSD64TR expression vector. RNA was transcribed from BamHI-linearized DNA using SP6 RNA polymerase (Message Machine®, Ambion®) and injected into Xenopus oocytes prepared as previously described (Goulding et al., 1992).

Patch clamp recordings were made from cell-free inside-out and outside-out patches 3-7 days after cRNA injection. Data were acquired using either an Axopatch 200A or 200B integrating patch clamp amplifier (Axon Instruments®, USA). The holding potential in all of these experiments was -40mV. For simplicity, only abbreviated descriptions of the solutions used are given in the text and in Brief Description of the Figures, full descriptions are given below.

The KCl-EGTA solution contained: 107 mM KCl, 5 mM NaCl, 10 mM HEPES (pH 7.4, KOH), 1 mM EGTA. The NaCl-EGTA solution contained 107 mM NaCl, 5 mM KCl, 10 mM HEPES (pH 7.4, NaOH), 1 mM EGTA. The KCl/NaCl-EGTA and KCl/NaCl-CaCl2 solutions contained: 82 mM KCl, 30 mM NaCl, 10 mM HEPES (pH 7.4, KOH) with either 1 mM EGTA or 1 mM CaCl2, respectively. In some experiments we replaced Cl with aspartate in the following KAspartate-EGTA solution: 107 mM K-Aspartate, 5 mM NaCl, 10 mM HEPES (pH 7.4, KOH), 1 mM EGTA. Where appropriate, Na-cAMP was included in the intracellular solution by iso-osmolar replacement of NaCl, while CsCl and BaCl2 were included in the indicated extracellular solutions by iso-osmotic replacement of NaCl or CaCl2, respectively. A Ag-AgCl ground wire was connected to the bath solution by a 3 M KCl agar bridge electrode. The largest uncompensated junction potential was measured to be 3.4 mV. Voltages have not been corrected to account for this offset. All recordings were obtained at room temperature (22-24 °C).

Voltage clamp protocols were applied using a P/N protocol to subtract uncompensated linear leak and capacitance using either pClamp® software (v 6.0, AXON INSTRUMENTS®, with N = 8) and a Pentium 100 mHz PC computer or the Pulse software (v 8.11, Heka, with N= 10) and a MACINTOSH CENTRIS® 650 computer. Unless otherwise indicated, data were low pass filtered at 1.25 kHz (8 pole Bessel filter, Frequency Devices 902) and digitized at 2.5 kHz using either a TL-1 DMA Interface (AXON INSTRUMENTS®) or an ITC-16 interface (INSTRUTECH CORP.®). Analyses were done using pClamp (v 6.0, AXON INSTRUMENTS®), Pulse (v 8.11, Heka) or Sigmaplot (v 4.0 SPSS Inc.).

The current-voltage relationship was obtained by measuring steady-state current values at the end of 3 s voltage steps (current averaged between 2.5 s to 2.95 s). The steady-state activation curve was determined from the amplitude of tail currents observed upon a subsequent depolarizing voltage step to -40 mV (current averaged between 4-10 ms following the return to -40 mV). Current values were plotted versus the hyperpolarization step voltage and fitted with the Boltzmann equation:

$$I(V) = A_1 + A_2 / \{1 + \exp[-(V-V_{1/2})/\text{slope}]\}$$

where A_1 is the offset, A_2 the amplitude, V is voltage in mV and $V_{1/2}$ is the activation mid-point voltage. The data and the fitted Boltzmann function were then normalized to the maximum amplitude of the fit. Activation time constants were determined by fitting single exponentials to the rising phase of the current after allowing for an initial lag.

To determine reversal potentials under different ionic conditions, inside-out patches held at -40 mV were stepped to -130 mV for 300 ms and then stepped back to a series of test potentials ranging from -60 to +20 mV in 5 mV

increments. The peak amplitude of the tail currents were measured by averaging the data between 2 and 4 ms following the step to the test potential. The sensitivity of the mBCNG-1 current to external Cs and Ba was determined using the outside-out patch clamp configuration and measuring the steady-state current values at the end of 1 s steps to -130 mV (current averaged between 800 ms to 990 ms). In both of these series of experiments, electrodes were coated with Sylgard to minimize pipette capacitance, and data were filtered at 2.5 kHz and sampled at 5 kHz.

Contamination from an endogenous Ca-activated Cl channel in the oocyte membrane (Barish, 1983) was minimized by including EGTA (1 mM) with no added calcium in the internal solution. Oocytes also contain a stretch-activated cation channel that can be recognized by its large single channel conductance (60 pS) (Yang and Sachs, 1990). Patches containing such channel activity were not studied.

EXAMPLE 5: Co-expression of BCNG channel subunits and candidate proteins.

BCNG proteins or BCNG-related proteins may form heteromultimeric proteins. In order to delineate the functional roles of novel BCNG subunits, novel BCNG subunits are coexpressed with BCNG subunits that have overlapping tissue distribution. Voltage-gated K^+ channels and cyclic nucleotide-gated channels both form heteromultimers. In some cases, the subunits can form complexes with completely distinct proteins (eg. KvLQT1 with MinK - (Barhanin, et al., 1996; HERG with MinK McDonald, et al., 1997); IrK6.1 and IrK6.2 with the sulphonylurea receptor - Isomoto, et al., 1996; Inagaki, et al., 1996). BCNG proteins may assemble with subunits such as MinK or ERG like subunits. Candidate proteins are

selected on the basis of overlapping tissue distribution and likelihood based on known functional properties. For example, Kv1.2 shows overlapping distribution with mBCNG-1 even at the subcellular level (Sheng, et al., 1994; Wang, et al., 1994).

Coexpression with polyA+ mRNA. If another protein can form a functional heteromultimer with the BCNG channel proteins, co-expression with size fractionated mRNA from tissue (eg. heart, brain, muscle or kidney) where the appropriate BCNG subunit is expressed (as shown by Northern blot analysis) should result in a unique current in electrophysiological currents when the BCNG RNA is coinjected with the mRNA from the tissue.

Alternative strategies to clone subunits that will modify functional properties of the expressed BCNG channels include low stringency homology screening of appropriate libraries using nucleotide probes derived from BCNG genes or PCR amplification from genomic or cDNA using degenerate oligonucleotides based on BCNG genes.

Yet another method to isolate other channel subunit proteins that may coassemble with identified BCNG family members is to use the yeast two hybrid system (Fields and Soug, 1989). This system was initially used to clone mBCNG-1 based on its interaction with the n-src SH3 domain (See, Example 1). Conserved cytoplasmic N- and C-terminal domains from BCNG channel proteins are used as the 'bait' in the yeast two hybrid system. N- and C-terminal fragments are subcloned in an appropriate plasmid (e.g. pEG202) (Zervos et al., 1983).

Sequences of BCNG family members: DNA and Predicted Amino Acid Sequences of Mouse and Human BCNG Clones

Mouse sequences

DNA and predicted amino acid sequence of mBCNG-1

These mouse sequences are the original DNA and predicted amino acid sequences obtained and are those in GenBank Accession Number AF028737.

mBCNG-1 DNA open reading frame

ATGGAAGGCGGCGGCAAACCCAACTCCGCGTCCAACAGCCGCGACGATGGCAACAG
CGTCTTCCCCTCCAAGGCGCCCGCGACGGGGCCGGTGGCGGCCGACAAGCGCCTGG
GGACCCCGCCGAGGGGCGGCGCGGCCGGGAAGGAACATGGCAACTCCGTGTGCTTC
AAGGTGGACGGCGGCGGAGGAGAGGAGCCGGCGGGCAGCTTCGAGGATGCCGAGGGG
CCCCGGCGGCAGTATGGTTTCATGCAGAGGCAGTTCACCTCCATGCTGCAGCCTGGG
GTCAACAAATTCTCCCTCCGCATGTTTGGGAGCCAGAAGGCGGTGGAGAAGGAGC
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GTTTTATTGGGATTTAATCATGCTTATAATGATGGTTGGAAATTTGGTCATCATA
CCAGTTGGAATCACGTTCTTCACAGAGCAGACGACAACACCGTGGATTATTTTCA
ACGTGGCATCCGATACTGTTTTCCTGTTGGACTTAATCATGAATTTTAGGACTGG
GACTGTCAATGAAGACAGCTCGGAAATCATCCTGGACCCTAAAGTGATCAAGATGA
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GCACTTCGTATCGTGAGGTTTACAAAAATTCTCAGTCTCTTGCGGTTATTACGCC
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TGACCTCGCCAGTGCTGTGGTGAGGATCTTCAACCTCATTGGCATGATGCTGCTT
CTGTGCCACTGGGATGGCTGTCTTCAGTTCCTGGTTCCCCTGCTGCAGGACTTCC
CACCAGATTGCTGGGTTTCTCTGAATGAAATGGTTAATGATTCCTGGGGAAAACA
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GAAGAAAATGTATTTCATCCAGCACGGTGTTGCTGGCGTTATCACCAAGTCCAGT
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AGGGCCGGCGCACTGCCAGTGTCCGAGCTGATACCTACTGTCGTCTTTACTCCCT
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CACAAGCCCTTCATAACACCAACCTGACCAAAGAAGTCAGGCCCCTTTCCGCCTC
GCAGCCTTCTCTGCCCCATGAGGTCTCCACTTTGATCTCCAGACCTCATCCCACT
GTGGGCGAATCCCTGGCCTCTATCCCTCAACCCGTGGCAGCAGTCCACAGCACTG
GCCTTCAGGCAGGGAGCAGGAGCACAGTGCCACAACGTGTCACCTTGTTCCGACA
GATGTCCTCGGGAGCCATCCCCCCCAACCGAGGAGTGCCTCCAGCACCCCCTCCA
CCAGCAGCTGTGCAGAGAGAGTCTCCCTCAGTCCTAAATACAGACCCAGATGCAG
AAAAACCCCGTTTTGCTTCGAATTTATGA

mBCNG-1 predicted amino acid sequence

MEGGGKPNSASNSRDDGNSVFPSKAPATGPVAADKRLGTPPRGGAAGKEHGNSVCFK
VDGGGGEEPAGSFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSLRMFGSQKAVEKE
QERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWIIF
NVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPVD
YIFLIVEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMT
YDLASAVVRIFNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGK
QYSYALFKAMSHMLCIGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQ
SLDSSRRQYQEKYKQVEQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILSEL
NDPLREEIVNFNCRKLVATMPLFANADPNFVTAMLSKLRFEVFQPGDYIIREGAV

GKKMYFIQHGVAGVITKSSKEMKLTDGSYFGEICLLTKGRRTASVRADTYCRLYS
LSVDNFNEVLEEYPMMRRAFETVAIDRLDRIGKKNSILLQKFQKDLNTGVFNNQE
NEILKQIVKHDREMVQAIPPINYPQMTALNCTSSTTTPTSRMRTQSPPVYTATSL
SHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPIQSPLATRTFHYASPTASQLS
LMQQPQQQLPQSQVQQTQTQTQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQ
QQQPQTPGSSTPKNEVHKSTQALHNTNLTKEVRPLSASQPSLPHEVSTLISRPHP
TVGESLASIPQPVAAVHSTGLQAGSRSTVPQRVTLFRQMSSGAIPPNRGVPPAPP
PPAAVQRESPSVLNTDPDAEKPRFASNL

DNA and predicted amino acid sequence of mBCNG-2

GenBank Accession Number AF064873.

mBCNG-2 DNA sequence

AAGTTCTCCCTGCGGATGTTCGGCAGCCAGAAGGCCGTGGAGCGCGAGCAGGAACG
CGTGAAGTCGGCGGGGGCCTGGATCATCCACCCCTACAGCGACTTCAGGTTCTACTG
GGACTTCACCATGCTGTTGTTCATGGTGGGAAATCTCATTATCATTCCCGTGGGCA
TCACTTTCTTCAAGGACGAGACCACCGCGCCCTGGATCGTCTTCAACGTGGTCTCG
GACACTTTCTTCCTCATGGACTTGGTGTTGAACTTCCGCACCGGCATTGTTATTG
AGGACAACACGGAGATCATCCTGGACCCCGAGAAGATAAAGAAGAAGTACTTGCGTA
CGTGGTTCGTGGTGGACTTCGTGTCATCCATCCCGGTGGACTACATCTTCCTCATA
GTGGAGAAGGGAATCGACTCCGAGGTCTACAAGACAGCGCGTGCTCTGCGCATCGT
GCGCTTCACCAAGATCCTCAGTCTGCTGCGGCTGCTGCGGCTATCACGGCTCATCC
GATATATCCACCAGTGGGAAGAGATTTTCCACATGACCTACGACCTGGCAAGTGCA
GTGATGCGCATCTGTAACCTGATCAGCATGATGCTACTGCTCTGCCACTGGGACGG
TTGCCTGCAGTTCCTGGTGCCCATGCTGCAAGACTTCCCCAGCGACTGCTGGGTG
TCCATCAACAACATGGTGAACCACTCGTGGAGCGAGCTCTACTCGTTCGCGCTCTT
CAAGGCCATGAGCCACATGCTGTGCATCGGCTACGGGCGGCAGGCGCCCGAGAGCAT
GACAGACATCTGGCTGACCATGCTCAGCATGATCGTAGGCGCCACCTGCTATGCC
ATGTTCATTGGGCACGCCACTGCGCTCATCCAGTCCCTGGATTCGTCACGGCGCC
AATACCAGGAGAAGTACAAGCAAGTAGAGCAATACATGTCCTTCCACAAACTGCC
CGCTGACTTCCGCCAGAAGATCCACGATTACTATGAACACCGGTACCAAGGGAAG
ATGTCTGATGAGGACAGCATCCTTGGGGAACTCAACGGGCCACTGCGTGAGGAGA
TTGTGAACTTCAACTGCCGGAAGCTGGTGGCTTCCATGCCGCTGTTTGCCAATGC
AGACCCCAATTTCGTCACAGCCATGCTGACAAAGCTCAAATTTGAGGTCTTCCAG
CCTGGAGATTACATCATCCGAGAGGGGACCATCGGGAAGAAGATGTACTTCATCC
AGCATGGGGTGGTGAGCGTGCTCACCAAGGGCAACAAGGAGATGAAGCTGTCGGA
TGGCTCCTATTTCGGGGAGATCTGCTTGCTCACGAGGGGCCGGCGTACGGCCAGC

GTGCGAGCTGACACCTACTGTCGCCTCTACTCACTGAGTGTGGACAATTTCAACG
AGGTGCTGGAGGAATACCCCATGATGCGGCGTGCCTTTGAGACTGTGGCTATTGA
CCGGCTAGATCGCATAGGCAAGAAGAACTCCACCTTGCTGCACAAGGTTCAGCAT
GATCTCAGCTCC

mBCNG-2 predicted amino acid sequence

KFSLRMFGSQKAVEREQERVKSAGAWIIHPYSDFRFYWDFTMLLFMVGNLIIIPV
GITFFKDETTAPWIVFNVVSDTFFLMDLVLNFRTGIVIEDNTEIILDPEKIKKKY
LRTWFVVDFVSSIPVDYIFLIVEKGIDSEVYKTARALRIVRFTKILSLLRLLRLS
RLIRYIHQWEEIFHMTYDLASAVMRICNLISMMLLLCHWDGCLQFLVPMLQDFPS
DCWVSINNMVNHSWSELYSFALFKAMSHMLCIGYGRQAPESMTDIWLTMLSMIVG
ATCYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYMSFHKLPADFRQKIHDYYEH
RYQGKMSDEDSILGELNGPLREEIVNFNCRKLVASMPLFANADPNFVTAMLTKLK
FEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGNKEMKLSDGSYFGEICLLTRG
RRTASVRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVAIDRLDRIGKKNSTLL
HKVQHDLSS

DNA and predicted amino acid sequence of mBCNG-3
GenBank Accession Number AF064874.

mBCNG-3 DNA sequence

TGCGAGCAGCCCTCGGCGGACACCGCTATCAAAGTGGAGGGAGGCGCGGCCGCCA
TCGACCATATCCTCCCCGAGGCCGAGGTGCGCCTGGGCCAAAGCGGCTTCATGCA
GCGCCAGTTCGGTGCCATGCTGCAACCTGGGGTCAACAAATTCTCCCTAAGGATG
TTCGGCAGCCAGAAAGCGGTGGAGCGCGAGCAGGAGAGGGTTAAGTCAGCAGGGT
TTTGGATTATCCACCCCTACAGTGACTTCAGATTTTACTGGGACCTGACGATGCT
GTTGCTGATGGTGGGGAATCTGATCATCATACCCGTGGGCATCACCTTCTTCAAG
GATGAGAACACCACACCCTGGATCGTCTTCAATGTGGTGTCAGACACATTCTTCC
TCATTGACTTGGTCCTCAACTTCCGCACGGGGATCGTGGTGGAGGACAACACAGA
AATCATCCTTGACCCGCAGAGGATCAAGATGAAGTACCTGAAAAGCTGGTTTGTG
GTAGATTTCATCTCCTCCATACCTGTCGAATACATTTTCCTTATAGTGGAGACTC
GCATTGACTCGGAGGTTTACAAAACCGCTAGGGCTGTGCGCATTGTCCGTTTCAC
TAAGATCCTCAGCCTCCTGCGCCTCTTGAGGCTTTCCCGCCTCATTCGATACATT
CATCAGTGGGAAGAGATTTTCCACATGACCTATGACCTGGCCAGCGCCGTGGTAC
GCATCGTGAACCTCATTGGCATGATGCTTCTGCTGTGTCACTGGGATGGCTGCCT
GCAGTTCCTAGTGCCCATGCTGCAGGACTTCCCCCATGACTGCTGGGTGTCCATC

AATGGCATGGTGAATAACTCCTGGGGGAAGCAGTATTCCTACGCCCTCTTCAAGG
CCATGAGCCACATGCTGTGCATTGGGTATGGACGGCAGGCACCCGTAGGCATGTC
TGACGTCTGGCTCACCATGCTCAGCATGATCGTGGGGGCCACCTGCTATGCCATG
TTCATCGGCCACGCCACTGCCCTCATCCAGTCGCTAGACTCCTCCCGGCGCCAGT
ACCAGGAGAAGTATAAACAGGTGGAGCAGTACATGTCTTTCCACAAGCTCCCGCC
TGACACCCGACAGCGCATCCATGACTACTATGAACACCGTTACCAAGGCAAGATG
TTTGATGAGGAAAGCATCCTGGGTGAGTTGAGTGAGCCACTTCGAGAGGAGATCA
TCAACTTTAACTGCCGAAAGCTGGTGGCATCCATGCCACTGTTTGCCAACGCAGA
TCCCAACTTTGTGACATCCATGCTGACCAAGTTGCGTTTCGAGGTCTTCCAGCCT
GGGGATTACATCATCCGCGAAGGCACCATCGGCAAGAAGATGTACTTTATCCAGC
ACGGCGTGGTCAGCGTGCTCACTAAGGGCAACAAAGAGACCAGGCTGGCTGATGG
CTCCTATTTTGGAGAGATCTGCTTGCTGACCCGGGGTCGGCGCACAGCCAGCGTC
AGAGCGGATACTTATTNCCGCCTCTACTCACTG

mBCNG-3 predicted amino acid sequence

CEQPSADTAIKVEGGAAAIDHILPEAEVRLGQSGFMQRQFGAMLQPGVNKFSLRM
FGSQKAVEREQERVKSAGFWIIHPYSDFRFYWDLTMLLLMVGNLIIIPVGITFFK
DENTTPWIVFNVVSDTFFLIDLVLNFRTGIVVEDNTEIILDPQRIKMKYLKSWFV
VDFISSIPVEYIFLIVETRIDSEVYKTARAVRIVRFTKILSLLRLLRLSRLIRYI
HQWEEIFHMTYDLASAVVRIVNLIGMMLLLCHWDGCLQFLVPMLQDFPHDCWVSI
NGMVNNSWGKQYSYALFKAMSHMLCIGYGRQAPVGMSDVWLTMLSMIVGATCYAM
FIGHATALIQSLDSSRRQYQEKYKQVEQYMSFHKLPPDTRQRIHDYYEHRYQGKM
FDEESILGELSEPLREEIINFNCRKLVASMPLFANADPNFVTSMLTKLRFEVFQP
GDYIIREGTIGKKMYFIQHGVVSVLTKGNKETRLADGSYFGEICLLTRGRRTASV
RADTYXRLYSL

DNA and predicted amino acid sequence of mBCNG-4

Reported are the complete DNA sequence of clone M28-EST, and the open reading frame (ORF) encoded between positions 632 and 871 of that DNA sequence. GenBank accession number AF064875.

mBCNG-4 DNA sequence

TTTTTGGGTTTTAAAATTTATTTTATTTTTAAAAGCGTCTCCGGANANTCTAGTG
CATGGCCAGGCTACAAGCTACTGGGCCAGCAACTCTGTAGGATTATTAATGACAA
AAATGCAAGGACCCCATAGTTGATGGAAACCCAGGGATGAAGCAGGGCTGTCCCA
CAGACTTAGGCTTTGTGGAGCTGTCTGAAAACCCAGGCTGTGGCTTTGGAAGAAG

TGCAGACAACCACTGCCCAGAGTGACTTAAGGTTCATACAACCATCCAGCCACCT
AAGCACCCCTACCTTCAAGCATCTTGCCAGTCCCACTTTGTGTCTGTTTAGCCTG
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GGGGTTGGAGTTAATGTGTAAAATAACTGAAAAAAACATCTGGGGCTGGCAAACC
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AGAGCAGTAAGGGACCTTATACCAGCTAATCGTTCATTCTCCCAAGTATAAGGAG
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GCTCTGAGCTCTTCCCCGCTTTCGCAGAGATCTGCCTGCTGACTCGAGGTCGGAG
AACAGCCAGTGTAAGGGCTGACACCTATTGTCGCCTCTACTCGCTCAGCGTGGAC
CACTTCAATGCGGTGCTTGAGGAGTTCCCAATGATGCGCAGGGCTTTTGAGACGG
TGGCCATGGACCGGCTTCGGCGCATCGGTGAGGCCTGTTTACTCTGTCTGCTCTG
GGTCCTGGCTGGGCCTCATCTCATGAGCCTAGCCCTGGTGCTTTGACACCACATC
CCAGCCCACCCAGTTCCAGTCCATGCCTCCAGCAGGCTGTTAGCACTGTTGCTCA
CTAGACTTAGCCCTAGCGAGAAATTGCCGTGGAGTGTCTCCCCAAACCCTCATTC
CCCGTGTCCTTCTGGGTACCAGTTCTTAACCTCACAATTTTTTATTGATA

mBCNG-4 predicted amino acid sequence

EICLLTRGRRTASVRADTYCRLYSLSVDHFNAVLEEFPMMRRAFETVAMDRLRRI
GEACLLCLLWVLAGPHLMSLALVL

Human Sequences:

DNA and predicted amino acid sequence of hBCNG-1

GenBank accession number AF064876.

hBCNG-1 DNA sequence

AAGGAGCAGGAAAGGGTTAAAACTGCAGGCTTCTGGATTATCCACCCTTACAGTGA
TTTCAGGTTTTACTGGGATTTAATAATGCTCATAATGATGGTTGGAAATCTAGTCAT
CATACCAGTTGGAATCACATTCTTTACAGAGCAAACAACAACACCATGGATTATTTT
CAATGTGGCATCAGATACAGTTTTCCTATTGGACCTGATCATGAATTTTAGGACT
GGGACTGTCAATGAAGACAGTTCTGAAATCATCCTGGACCCCAAAGTGATCAAGA
TGAATTATTTAAAAAGCTGGTTTGTGGTTGACTTCATCTCATCCATCCCAGTGGATT
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TCAAGGTTAATTAGATACATACATCAATGGGAAGAGATATTCCACATGACATATG
ATCTCGCCAGTGCAGTGGTGAGAATTTTTAATCTCATCGGCATGATGCTGCTCCTGT
GCCACTGGGATGGTTGTCTTCAGTTCTTAGTACCACTACTGCAGGACTTCCCACC
AGATTGCTGGGTGTCTTTAAATGAAATGGTTAATGATTCTTGGGGAAAGCAGTAT
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GATTCTTCGAGGCGGCAGTATCAAGAGAAGTATAAGCAAGTGGAACAATACATGT
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AAAATGTATTTCATTCAACACGGTGTTGCTGGTGTCATTACAAAATCCAGTAAAG
AAATGAAGCTGACAGATGGCTCTTACTTTGGAGAGATTTGCCTGCTGACCAAAGG
ACGTCGTACTGCCAGTGTTCGAGCTGATACATATTGTCGTCTTTACTCACTTTCC
GTGGACAATTTCAACGAGGTCCTGGAGGAATATCCAATGATGAGGAGAGCCTTTG
AGACAGTTGCCATTGACCGACTAGATCGAATAGGAAAGAAAAATTCAATTCTTCT
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TCAATTATCCTCAAATGACAACCCTGAATTCCACATCGTCTACTACGACCCCGAC
CTCCCGCATGAGGACACAATCTCCACCGGTGTACACAGCGACCAGCCTGTCTCAC
AGCAACCTGCACTCCCCCAGTCCCAGCACACAGACCCCCCAGCCATCAGCCATCC
TGTCACCCTGCTCCTACACCACCGCGGTCTGCAGCCCTCCTGTACAGAGCCCTCT
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CAACAGCAGCCGCAGCAGCAGGTACAGCAGTCCCAGCCGCCGCAGACTCAGCCAC
AGCAGCCGTCCCCGCAGCCACAGACACCTGGCAGCTCCACGCCGAAAAATGAAGT
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TTTTCCGCCTGGCAGCCNTCGCTGCCCCATGAGGTGTCCATTTTGATTTCCAGAC
CCATCCCACTGTGGGGGAGTCCCTGGCCTCCATCCCTCAACCCGTGACGGCGGTC
CCCGGAACGGGCCTTCAGGCAGGGGGCAGGAGCACTGTCCCGCAGCGCGTCACCT
TTTTCCGACAGATGTNGTCGGGAGCCATCCCCCCGAACCGAGGAGTCCTTCCAGC
ACCCCTTCCACTTATCACACCCCATCCTAAAAAA

hBCNG-1 predicted amino acid sequence

KEQERVKTAGFWIIHPYSDFRFYWDLIMLIMMVGNLVIIPVGITFFTEQTTTPWII
FNVASDTVFLLDLIMNFRTGTVNEDSSEIILDPKVIKMNYLKSWFVVDFISSIPV

DYIFLIVEKGMDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTY
DLASAVVRIFNLIGMMLLLCHWDGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYS
YALFKAMSHMLCIGYGAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLD
SSRRQYQEKYKQVEQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILNELNDP
LREEIVNFNCRKLVATMPLFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKK
MYFIQHGVAGVITKSSKEMKLTDGSYFGEICLLTKGRRTASVRADTYCRLYSLSV
DNFNEVLEEYPMMRRAFETVAIDRLDRIGKKNSILLQKFQKDLNTGVFNNQENEI
LKQIVKHDREMVQAIAPINYPQMTTLNSTSSTTTPTSRMRTQSPPVYTATSLSHS
NLHSPSPSTQTPQPSAILSPCSYTTAVCSPPVQSPLAARTFHYASPTASQLSLMQ
QQPQQQVQQSQPPQTQPQQPSPQPQTPGSSTPKNEVHKSTQALHNTNLTREVRPF
SAWQPSLPHEVSILISRPHPTVGESLASIPQPVTAVPGTGLQAGGRSTVPQRVTF
FRQMXSGAIPPNRGVLPAPLPLITPHPKK

DNA and predicted amino acid sequence of hBCNG-2

GenBank accession number AF064877.

hBCNG-2 DNA sequence

GCGAGGAGGCGGGCCCGGCGGGGGAGCCGCGCGGCAGCCAGGCCAGCTTCATGCAG
CGCCAGTTCGGCGCGCTCCTGCAGCCGGGCGTCAACAAGTTCTCGCTGCGGATGTT
CGGCAGCCAGAAGGCCGTGGAGCGCGAGCAGGAGCGCGTCAAGTCGGCGGGGGCC
TGGATCATCCACCCGTACAGCGACTTCAGGTTCTACTGGGACTTCACCATGCTGCTG
TTCATGGTGGGAAACCTCATCATCATCCCAGTGGGCATCACCTTCTTCAAGGATG
AGACCACTGCCCCGTGGATCGTGTTCAACGTGGTCTCGGACACCTTCTTCCTCATGG
ACCTGGTGTTGAACTTCCGCACCGGCATTGTGATCGAGGACAACACGGAGATCAT
CCTGGACCCCGAGAAGATCAAGAANAAGTATCTGCGCACGTGGTTCGTGGTGGTCTT
CGTGTCCTCCATCCCCGTGGACTACATCTTCCTTATCGTGGAGAAGGGCATTGACT
CCGAGGTCTACAAGACGGCACGCGCCCTGCGCATCGTGCGCTTCACCAAAATCCTCA
GCCTCCTGCGGCTGCTGCGCCTCTCACGCCTGATCCGCTACATCCATCAGTGGGA
GGAGATCTTCCACATGACCTATGACCTGGCCAGCGCGGTGATGAGGATCTGCAAT
CTCATCAGCATGATGCTGCTGCTCTGCCACTGGGACGGCTGCCTGCAGTTCCTGGTG
CCTATGCTGCAGGACTTCCCGCGCAACTGCTGGGTGTCCATCAATGGCATGGTGAAC
CACTCGTGGAGTGAACTGTACTCCTTCGCACTCTTCAAGGCCATGAGCCACATGC
TGTGCATCGGGTACGGCCGGCAGGCGCCCGAAAGCATGACGGACATCTGGCTGACCA
TGCTCAGCATGATTGTGGGTGCCACCTGCTACGCCATGTTCATCGGCCACGCCACTG
CCCTCATCCAGTCGCTGGACTCCTCGCGGCGCCAGTACCAGGAGAAGTACAAGCA
GGTGGAGCAGTACATGTCCTTCCACAAGCTGCCAGCTGACTTCCGCCAGAAGATC
CACGACTACTATGAACACCGTTACCAGGGCAAGATGTTTGACGAGGACAGCATCCTG

GGCGAGCTCAACGGGCCCCTGCGGGAGGAGATCGTCAACTTCAACTGCCGGAAGCTG
GTGGCCTCCATGCCGCTGTTCGCCAACGCCGACCCCAACTTCGTCACGGCCATGC
TGACCAAGCTCAAGTTCGAGGTCTTCCAGCCGGGTGACTACATCATCCGCGAAGG
CACCATCGGGAAGAAGATGTACTTCATCCAGCACGGCGTGGTCAGCGTGCTCACT
AAGGGCAACAAGGAGATGAAGCTGTCCGATGGCTCCTACTTCGGGGAGATCTGCC
TGCTCACCCGGGGCCGCCGCACGGCGANCGTGCGGGCTGACACCTACTGCCGCCT
CTATTCCCTGAGCGTGGACAACTTCAACGAAGTGCTGGAGGAGTACCCCATGATG
CGGCGCGCTTTCGAGACGGTGGCCATCGACCGCCTGGACCGCATCGGCAAGAAGA
ATTCCATCCTCCTGCACAAGGTGCAGCATGACCTCAACTCGGGCGTATTCAACAA
CCAGGAGAACGCCATCATCCAGGAGATCGTCAAGTACGACCGCGAGATGGTGCAG
CAGGCCGAGCTGGGTCAGCGCGTGGGCTTTTTCCCGCCGCCGCCGCCGCCGCCGC
AGGTCACTTCGGCCATCGCCACGCTGCAGCAGGCGGCGGCCATGAGCTTCTGCCC
GCAGGTGGC

hBCNG-2 predicted amino acid sequence

EEAGPAGEPRGSQASFMQRQFGALLQPGVNKFSLRMFGSQKAVEREQERVKSAGA
WIIHPYSDFRFYWDFTMLLFMVGNLIIIPVGITFFKDETTAPWIVFNVVSDTFFL
MDLVLNFRTGIVIEDNTEIILDPEKIKXKYLRTWFVVVFVSSIPVDYIFLIVEKG
IDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVMR
ICNLISMMLLLCHWDGCLQFLVPMLQDFPRNCWVSINGMVNHSWSELYSFALFKA
MSHMLCIGYGRQAPESMTDIWLTMLSMIVGATCYAMFIGHATALIQSLDSSRRQY
QEKYKQVEQYMSFHKLPADFRQKIHDYYEHRYQGKMFDEDSILGELNGPLREEIV
NFNCRKLVASMPLFANADPNFVTAMLTKLKFEVFQPGDYIIREGTIGKKMYFIQH
GVVSVLTKGNKEMKLSDGSYFGEICLLTRGRRTAXVRADTYCRLYSLSVDNFNEV
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YDREMVQQAELGQRVGFFPPPPPPPQVTSAIATLQQAAAMSFCPQVA

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What is claimed is:

1. An isolated nucleic acid encoding a BCNG protein or a portion thereof.

2. An isolated nucleic acid encoding a BCNG-related protein or a portion thereof.

3. The nucleic acid of claim 1, wherein the BCNG protein is encoded by the sequence shown in mBCNG-1 (ATCC Accession No. 209781) (Seq.ID.No.:1), mBCNG-2 (Seq.ID.No.:5), mBCNG-3 (Seq.ID.No.:9), mBCNG-4 (Seq.ID.No.:11), hBCNG-1 (ATCC Accession No. 209827) (Seq.ID.No.:3) or hBCNG-2 (Seq.ID.No.:7).

4. The nucleic acid of claim 1 or 2, wherein the nucleic acid is DNA or RNA.

5. The nucleic acid of claim 1 or 2, wherein the nucleic acid is cDNA.

6. The nucleic acid of claim 5, wherein the cDNA has the nucleotide sequence shown in SEQ. ID. No.: 1 for mBCNG-1 (ATCC Accession No. 209781) , SEQ. ID. No.: 3 for hBCNG-1, SEQ. ID. No.: 5 for mBCNG-2, SEQ. ID. No.: 7 for hBCNG-2, SEQ. ID. No.: 9 for mBCNG-3, or SEQ. ID. No.:11 for mBCNG-4.

7. A vector comprising the nucleic acid of claim 1 or 2.

8. The vector of claim 7, wherein the vector comprises viral or plasmid DNA.

9. An expression vector comprising the nucleic acid of claim 1 or 2 and regulatory elements.

10. A host vector system which comprises the expression vector of claim 9 in a suitable host.

11. A vector, which comprises cDNA encoding mBCNG-1 (ATCC Accession No. 209781).

12. A vector, which comprises cDNA encoding hBCNG-1 (ATCC Accession No. 209827).

13. The host vector system of claim 10, wherein the suitable host is a bacterial cell, a eukaryotic cell, a mammalian cell or an insect cell.

14. An isolated BCNG protein.

15. An isolated BCNG-related protein.

16. The protein of claim 14, wherein the BCNG protein has substantially the same amino acid sequence shown in Seq.ID.No.:2 for mBCNG-1 (Figure 8A), Seq.ID.No.:6 for mBCNG-2 (Figure 8A), Seq.ID.No.:10 for mBCNG-3 (Figure 8A), Seq.ID.No.12 for mBCNG-4 (Figure 8A), Seq.ID.No.:4 for hBCNG-1 (Figure 8A), Seq.ID.No.:8 for hBCNG-2 (Figure 8A) or a portion thereof.

17. The protein of claim 15, wherein the BCNG-related protein has substantial homology to the amino acid sequence shown in Seq.ID.No.:2 mBCNG-1 (Figure 8A), Seq.ID.No.:6 for mBCNG-2 (Figure 8A), Seq.ID.No.:10 for mBCNG-3 (Figure 8A), Seq.ID.No.12 for mBCNG-4 (Figure 8A), Seq.ID.No.:4 for hBCNG-1 (Figure 8A),

Seq.ID.No.:8 for hBCNG-2 (Figure 8A) or a portion thereof.

18. A composition comprising a nucleic acid, encoding a BCNG protein or a BCNG-related protein or a portion thereof and a carrier.

19. The composition of claim 18, wherein the nucleic acid comprises substantially the same coding sequence as the coding sequence shown in SEQ. ID. No.: 1 for mBCNG-1, SEQ. ID. No.: 3 for hBCNG-1, SEQ. ID. No.: 5 for mBCNG-2, SEQ. ID. No.: 7 for hBCNG-2, SEQ. ID. No.: 9 for mBCNG-3, SEQ. ID. No.:11 for mBCNG-4 or portion thereof.

20. A composition comprising a BCNG protein or a BCNG-related protein or portion thereof and a carrier.

21. The composition of claim 20, wherein the BCNG protein or BCNG-related protein comprises substantially the same amino acid sequence as the amino acid sequence shown in Seq.ID.No.:2 mBCNG-1 (Figure 8A), Seq.ID.No.:6 for mBCNG-2 (Figure 8A), Seq.ID.No.:10 for mBCNG-3 (Figure 8A), Seq.ID.No.12 for mBCNG-4 (Figure 8A), Seq.ID.No.:4 for hBCNG-1 (Figure 8A), Seq.ID.No.:8 for hBCNG-2 (Figure 8A) or a portion thereof.

22. A nucleic acid probe capable of specifically hybridizing with a nucleic acid encoding a BCNG protein or BCNG-related protein.

23. A nucleic acid probe capable of specifically hybridizing with the nucleic acid of claim 1.

24. The nucleic acid probe of claim 22, wherein the probe comprises a sequence selected from the following: Seq.ID.No:13, Seq.ID.No:14, Seq.ID.No:15, Seq.ID.No:16, Seq.ID.No:17, Seq.ID.No:18, Seq.ID.No:19, Seq.ID.No:20, Seq.ID.No:21, Seq.ID.No:21, Seq.ID.No:22, Seq.ID.No:23, Seq.ID.No:24, Seq.ID.No:25, Seq.ID.No:26, Seq.ID.No:27, Seq.ID.No:28, Seq.ID.No:29, Seq.ID.No:30, Seq.ID.No:31, Seq.ID.No:32, Seq.ID.No:33, Seq.ID.No:34, Seq.ID.No:35, or Seq.ID.No:36.

25. A method for identifying a nucleic acid in a sample which encodes a BCNG protein or a BCNG-related protein which comprises:

(a) contacting the sample with a nucleic acid probe capable of specifically hybridizing with nucleic acid encoding a BCNG protein or a BCNG-related protein under conditions permissive to the formation of a complex between the nucleic acid probe and the nucleic acid encoding the BCNG protein or the BCNG-related protein in the sample;

(b) determining the amount of complex formed in step (a); and

(c) comparing the amount of complex determined in step(b) with the amount of complex formed using an arbitrary sequence, a greater amount of complex formed with the BCNG-specific probe indicating the presence of a nucleic acid encoding a BCNG protein or a BCNG-related protein in the sample.

26. The method of claim 25, step (a) further comprising amplifying the nucleic acid molecule encoding the

BCNG protein or the BCNG-related protein.

27. The method of claim 26, wherein the amplification comprises contacting the nucleic acid molecule from the sample with at least one amplification primer capable of specifically hybridizing to mBCNG-1 (Seq.ID.No.:1), mBCNG-2 (Seq.ID.No.:5), mBCNG-3 (Seq.ID.No.:9), mBCNG-4 (Seq.ID.No.:11), hBCNG-1 (Seq.ID.No.:3) or hBCNG-2 (Seq.ID.No.:7) under conditions suitable for polymerase chain reaction.

28. The method of claim 26, wherein the amplified nucleic acid molecule encoding the BCNG protein or the BCNG-related protein is detected by size fractionation.

29. The method of claim 25, further comprising isolating the complex by size fractionation.

30. The method of claim 25, wherein the nucleic acid probe is labeled with a detectable marker.

31. The method of claim 30, wherein the detectable marker is a radiolabeled molecule, a fluorescent molecule, an enzyme, a ligand, or a magnetic bead.

32. The method of claim 25, wherein the probe comprises a sequence selected from the following: Seq.ID.No:13, Seq.ID.No:14, Seq.ID.No:15, Seq.ID.No:16, Seq.ID.No:17, Seq.ID.No:18, Seq.ID.No:19, Seq.ID.No:20, Seq.ID.No:21, Seq.ID.No:21, Seq.ID.No:22, Seq.ID.No:23, Seq.ID.No:24, Seq.ID.No:25, Seq.ID.No:26, Seq.ID.No:27, Seq.ID.No:28, Seq.ID.No:29, Seq.ID.No:30, Seq.ID.No:31, Seq.ID.No:32,

Seq.ID.No:33, Seq.ID.No:34, Seq.ID.No:35, or Seq.ID.No.36.

33. The method of claim 25, wherein the nucleic acid probe is capable of specifically hybridizing to mBCNG-1 (Seq.ID.No.:1), mBCNG-2 (Seq.ID.No.:5), mBCNG-3 (Seq.ID.No.:9), mBCNG-4 (Seq.ID.No.:11), hBCNG-1 (Seq.ID.No.:3) or hBCNG-2 (Seq.ID.No.:7).

34. An isolated nucleic acid, previously unknown, identified by the method of claim 25.

35. A method for testing whether a compound affects the expression of a BCNG protein or a BCNG-related protein which comprises:

(a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound;

(b) determining the amount of expression of BCNG protein or BCNG-related protein in the sample; and

(c) comparing the amount of BCNG protein or BCNG-related protein expression determined in step (b) with the amount determined in the absence of the compound.

36. A method for identifying a compound capable of interacting with a BCNG protein or a BCNG-related protein which comprises:

(a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound under conditions permissive to formation of a complex between the compound and the BCNG protein or the BCNG-related protein;

(b) determining the amount of complex formed between the compound and the BCNG protein or the BCNG-related protein;

(c) comparing the amount of complex formed in step (b) with the amount formed in the absence of the compound, a greater amount of complex formed in the presence of the compound indicating the presence of a compound capable of interacting with a BCNG protein or a BCNG-related protein.

37. A method for identifying a compound capable of modulating BCNG protein or BCNG-related protein activity which comprises:

(a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound;

(b) determining the amount of activity of the BCNG protein or BCNG-related protein in the sample; and

(c) comparing the amount of activity of the BCNG protein or the BCNG-related protein determined in step (b) with the amount determined in the absence of the compound, an increase or decrease in activity indicating the presence of a compound capable of modulating the activity of the BCNG protein or the BCNG-related protein.

38. The method of claim 37, step (a) comprising first introducing the nucleic acid encoding a BCNG protein or a BCNG-related protein into an expression system and causing the expression system to express the nucleic acid under conditions whereby a BCNG protein or a BCNG-related protein is produced.

(b) determining the amount of complex formed between the compound and the BCNG protein or the BCNG-related protein;

(c) comparing the amount of complex formed in step (b) with the amount formed in the absence of the compound, a greater amount of complex formed in the presence of the compound indicating the presence of a compound capable of interacting with a BCNG protein or a BCNG-related protein.

37. A method for identifying a compound capable of modulating BCNG protein or BCNG-related protein activity which comprises:

(a) contacting a sample which expresses a BCNG protein or a BCNG-related protein with a compound;

(b) determining the amount of activity of the BCNG protein or BCNG-related protein in the sample; and

(c) comparing the amount of activity of the BCNG protein or the BCNG-related protein determined in step (b) with the amount determined in the absence of the compound, an increase or decrease in activity indicating the presence of a compound capable of modulating the activity of the BCNG protein or the BCNG-related protein.

38. The method of claim 37, step (a) comprising first introducing the nucleic acid encoding a BCNG protein or a BCNG-related protein into an expression system and causing the expression system to express the nucleic acid under conditions whereby a BCNG protein or a BCNG-related protein is produced.

39. The method of claim 37, wherein step (b) comprises measuring the channel electrical current or intracellular calcium level in the presence of the compound.

40. The method of claim 38, wherein the expression system comprises a cultured host cell.

41. A compound, previously unknown, identified by the method of claim 35, 36, or 37.

42. The method of claim 25, 35, 36, or 37, wherein the BCNG protein or the BCNG-related protein comprises mBCNG-1 (Seq.ID.No.:2), mBCNG-2 (Seq.ID.No.:6), mBCNG-3 (Seq.ID.No.:10), mBCNG-4 (Seq.ID.No.:12), hBCNG-1 (Seq.ID.No.:4) or hBCNG-2 (Seq.ID.No.:8) or a portion thereof.

43. The method of claim 25, 35, 36, or 37, wherein the sample comprises a cell, cell lysate or cell-free translation.

44. The method of claim 43, wherein the cell is a cardiac cell, a kidney cell, a hepatic cell, an airway epithelial cell, a muscle cell, a neuronal cell, a glial cell, a microglial cell, an endothelial cell, a mononuclear cell, a tumor cell, a mammalian cell, an insect cell, or a *Xenopus* oocyte.

45. A compound, previously unknown, identified by the method of claim 35, 36, or 37.

46. The method of claim 35, 36 or 37, wherein the

compound is a peptide, a peptidomimetic, a nucleic acid, a polymer, or a small molecule.

47. The method of claim 35, 36 or 37, wherein the compound is bound to a solid support.

48. The method of claim 25, 35, 36, or 37, wherein the BCNG protein or the BCNG-related protein is ion channel protein or an ion channel subunit protein.

49. The method of claim 35, 36, or 37, wherein the compound is an agonist or antagonist of ion channel activity

50. The method of claim 37 wherein the modulation is increased ion flow rate or decreased ion flow rate.

51. The method of claim 37 wherein the modulation is increased ion permissivity or decreased ion permissivity

52. A method of modulating BCNG or BCNG-related protein activity in a sample, comprising contacting the sample with the compound of claim 41.

53. A method of treating a condition in a subject which comprises administering to the subject an amount of the compound of claim 41, effective to treat the condition.

54. A pharmaceutical composition which comprises the compound of claim 41 and a pharmaceutically acceptable carrier.

55. The pharmaceutical composition of claim 54, wherein

the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, a nonaqueous solution or a solid carrier.

56. A method for treating a condition in a subject which comprises administering to the subject an amount of the pharmaceutical composition of claim 54, effective to treat the condition in the subject.

57. The method of claim 53 or 56, wherein the condition is a neurological, renal, pulmonary, hepatic, or cardiovascular condition.

58. The method of claim 57, wherein the condition is epilepsy, Alzheimer's Disease, Parkinson's Disease, long QT syndrome, sick sinus syndrome, age-related memory loss, cystic fibrosis, sudden death syndrome or a pacemaker rhythm dysfunction.

59. The method of claim 53 or 56, wherein the subject is a human.

60. An antibody which binds specifically to the protein of claim 14 or 15.

61. A cell capable of producing the antibody of claim 60.

62. A method of identifying the protein of claim 14 or 15 in a sample comprising:

a) contacting the sample with the antibody of claim 58 under conditions permissive to the formation of a complex between the antibody and the protein;

b) determining the amount of complex formed; and

c) comparing the amount of complex formed in step (b) with the amount of complex formed in the absence of the antibody, the presence of an increased amount of complex formed in the presence of the antibody indicating identification of the protein in the sample.

FIG. 1A

```
                                                                    50
MEGGGKPNSASNSRDDGNSVFPSKAPATGPVAADKRLGTPPRGGAAGKEH

                                                                   100
GNSVCFKVDGGGGEEPAGSFEDAEGPRRQYGFMQRQFTSMLQPGVNKFSL

                                          S1                       150
RMFGSQKAVEKEQERVKTAGFWIIHPYSDERFYWDLIMLIMMVGNLVIIP

                    S2                                             200
VGITFFTEQTTTPWIIFNVASDTVFLLDLIMNFRTGTVNEDSSEIILDPK

               S3                                        250
VIKMNYLKSWFVVDFISSIPVDYIFLIVEKGMDSEVYKTARALRIVRFTK

          S4                                        300
ILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIFNLIGMMLLLCHW

  S5                        *                 P     350
DGCLQFLVPLLQDFPPDCWVSLNEMVNDSWGKQYSYALFKAMSHMLCIGY

          S6                                        400
GAQAPVSMSDLWITMLSMIVGATCYAMFVGHATALIQSLDSSRRQYQEKY

                                                     450
KQVEQYMSFHKLPADMRQKIHDYYEHRYQGKIFDEENILSELNDPLREEI

                                                     500
VNFNCRKLVATMPLFANADPNFVTAMLSKLRFEVFQPGDYIIREGAVGKK
                          ----------------------------
                                                     550
MYFIQHGVAGVITKSSKEMKLTDGSYFGEICLLTKGRRTASVRADTYCRL
-------------------------------------------------------
                                                     600
YSLSVDNFNEVLEEYPMMRRAFETVAIDRLDRIGKKNSILLQKFQKDLNT
-------------------------------------------------------
                                  ..   .           .  650
GVFNNQENEILKQIVKHDREMVQAIPPINYPQMTALNCTSSTTTPTSRMR
--
    ..                  . .   . .     .          .. 700
TQSPPVYTATSLSHSNLHSPSPSTQTPQPSAILSPCSYTTAVCSPPIQSP

                .           .   .                    750
LATRTFHYASPTASQLSLMQQPQQQLPQSQVQQTQTQTQQQQQQQQQQQQ

                                    .   .   .        800
QQQQQQQQQQQQQQQQQQQQQQQQQQQPQTPGSSTPKNEVHKSTQALHNTNL

      .     .   .         . .           . .          850
TKEVRPLSASQPSLPHEVSTLISRPHPTVGESLASIPQPVAAVHSTGLQA

      .                   ..    ..  ....       .    900
GSRSTVPQRVTLFRQMSSGAIPPNRGVPPAPPPPAAVQRESPSVLNTDPD

      .
AEKPRFASNL*
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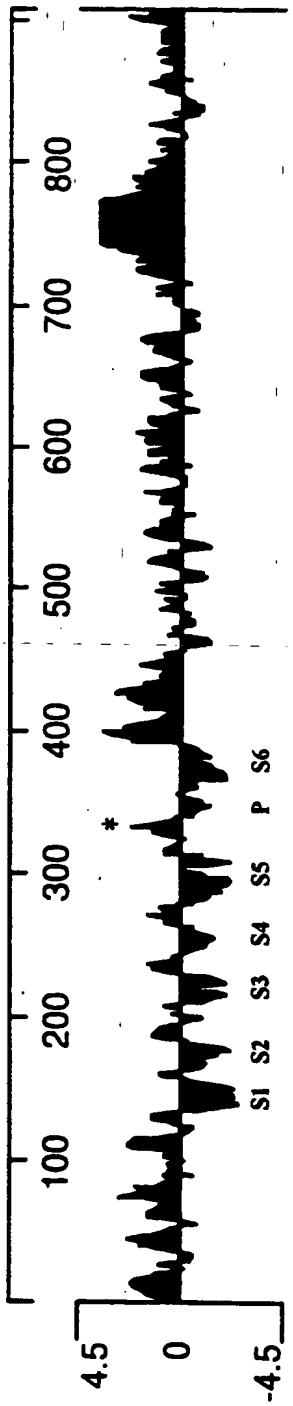


FIG. 1B

FIG. 1C

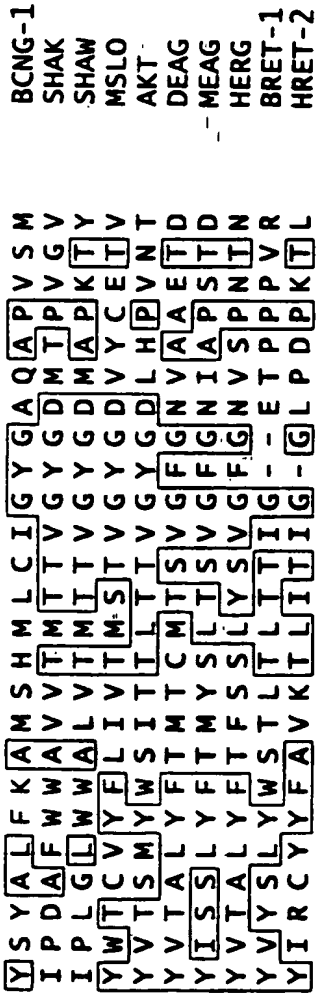


FIG. 1D

FIG. 2A

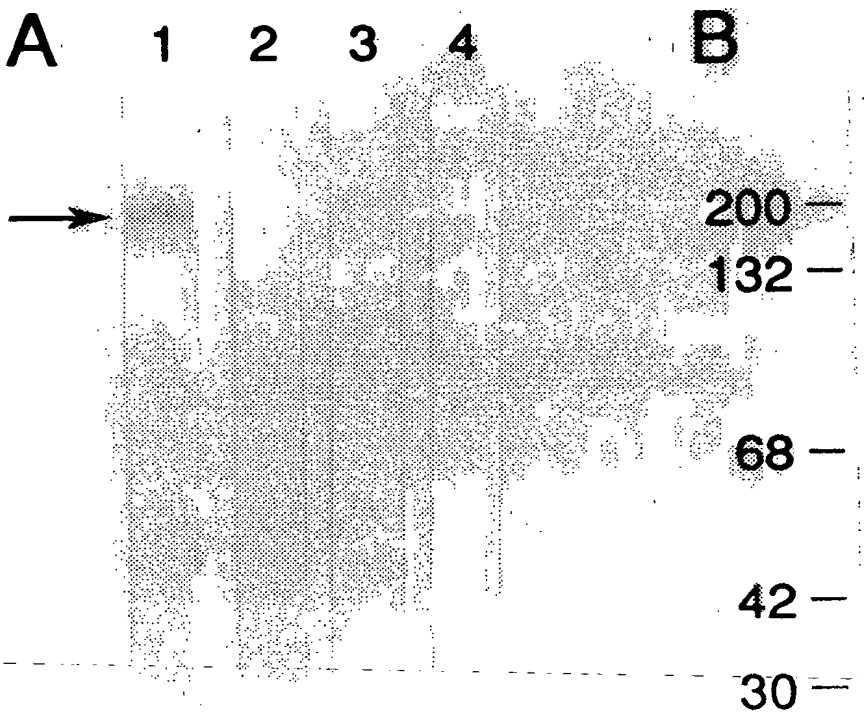


FIG. 2B

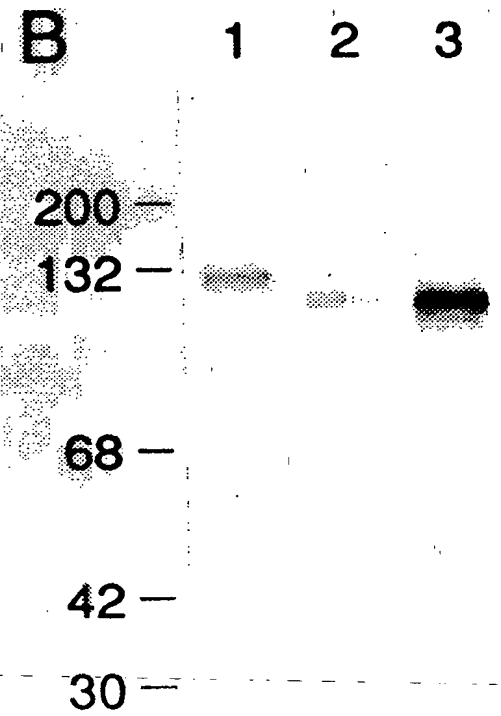
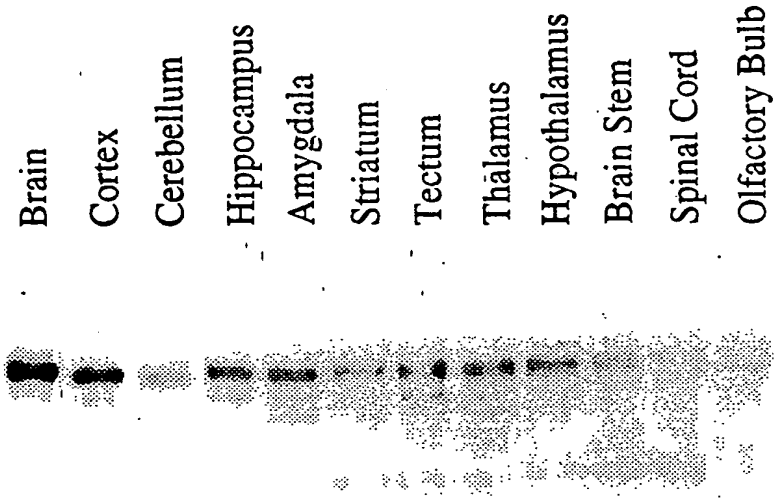
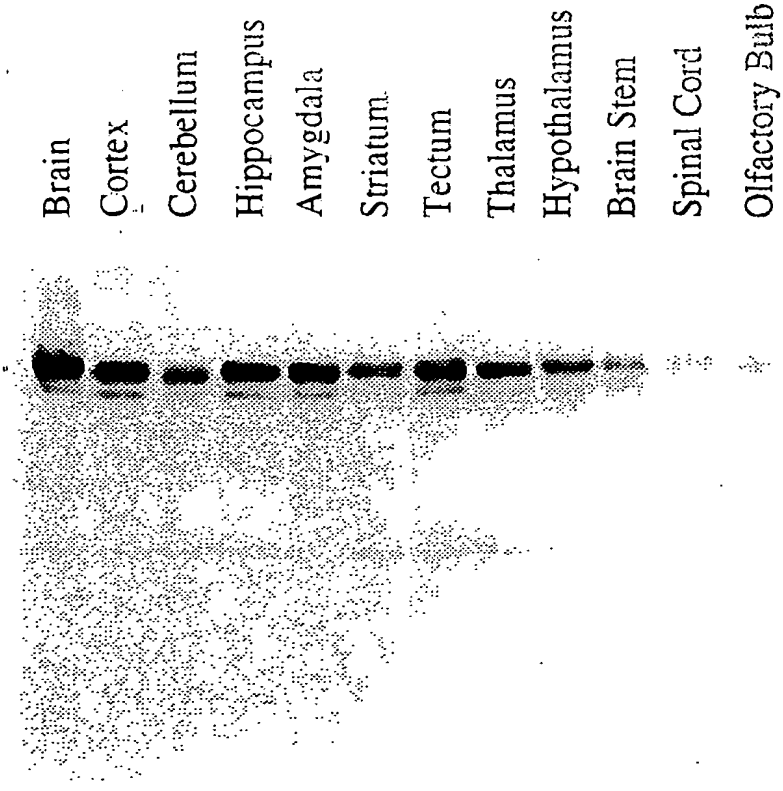


FIG. 2C



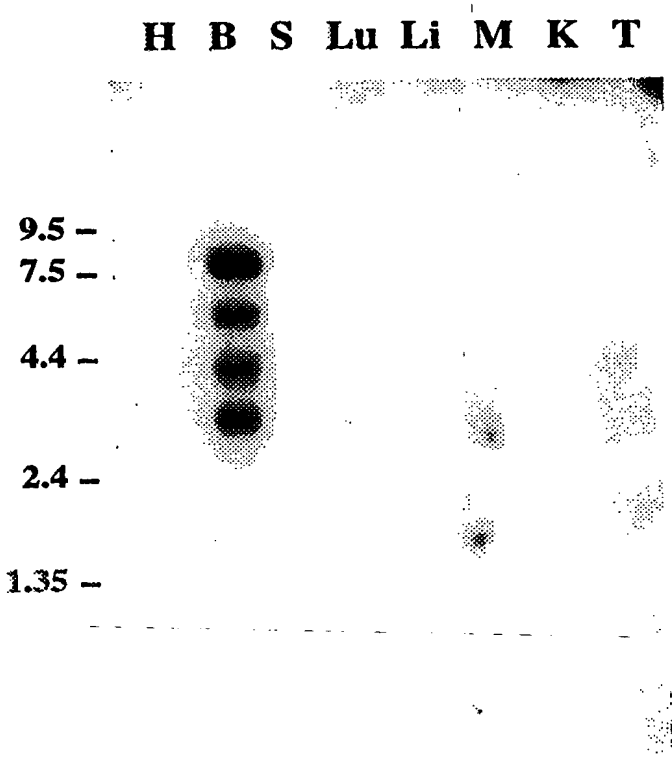
αq1

FIG. 2D



αq2

FIG. 3



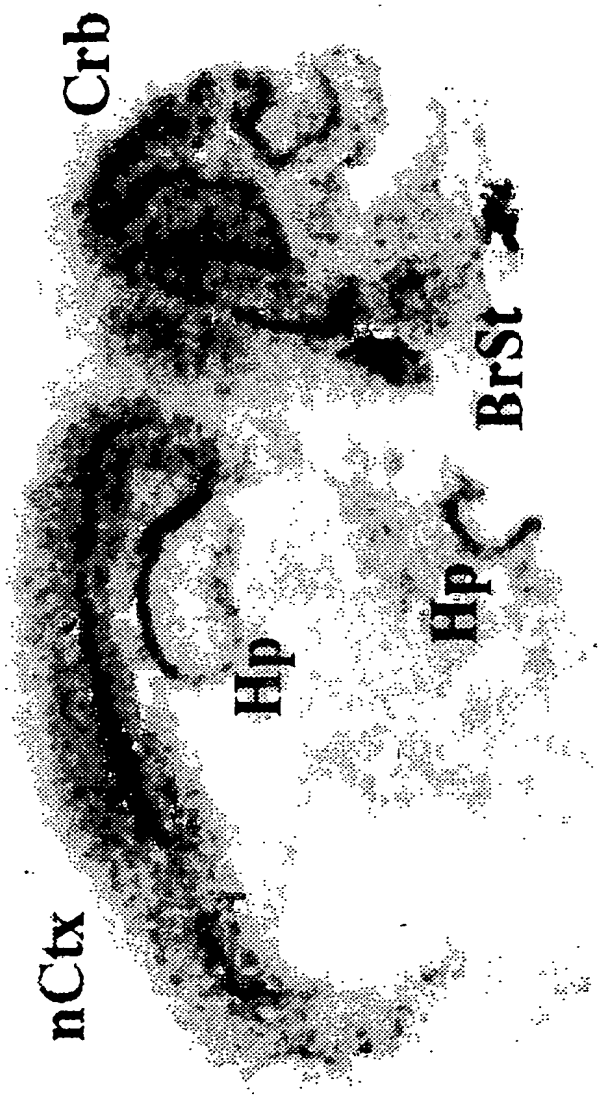


FIG. 4

FIG. 5B

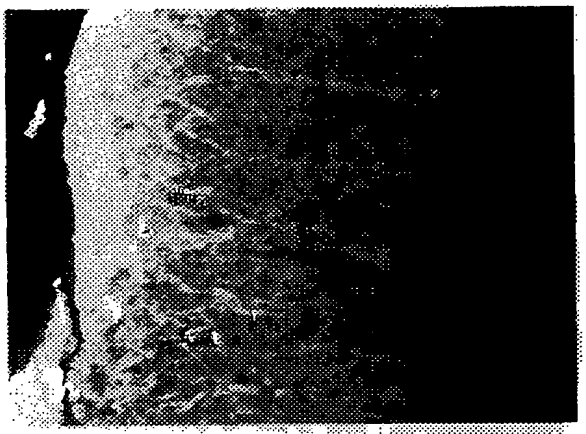


FIG. 5D

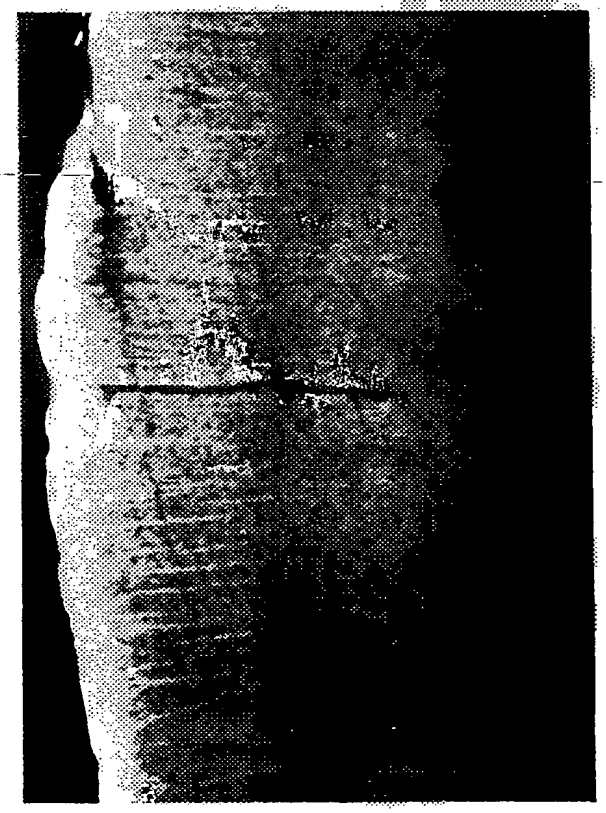


FIG. 5A

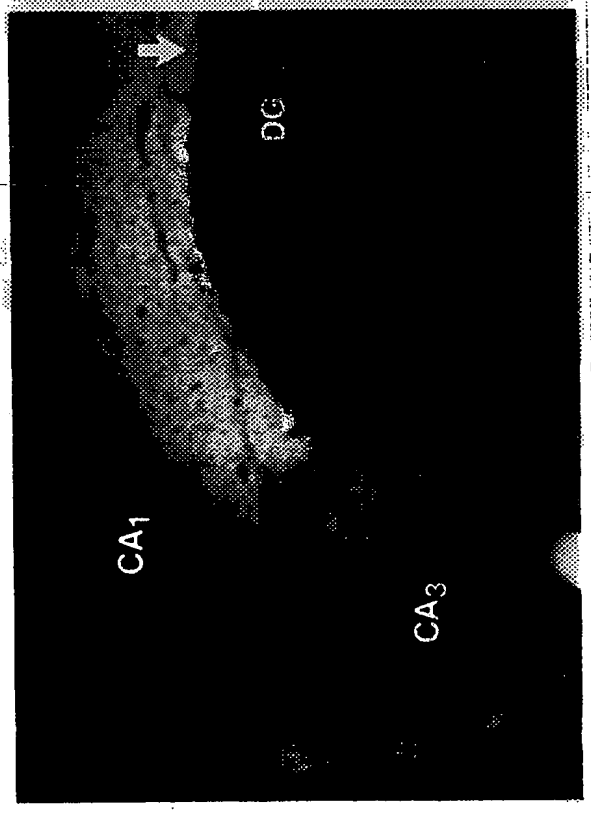


FIG. 5C

FIG. 5F

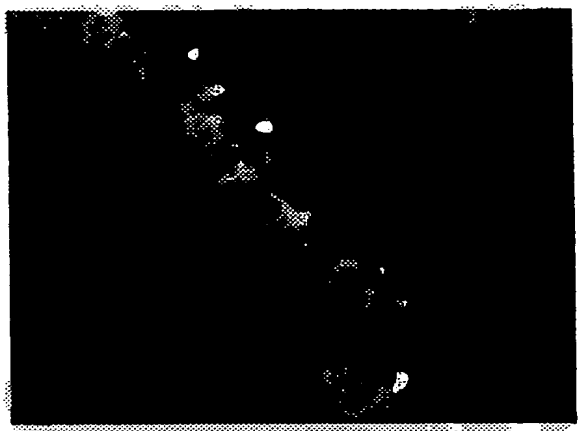
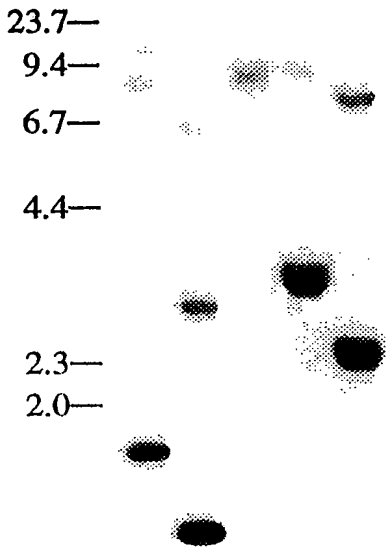


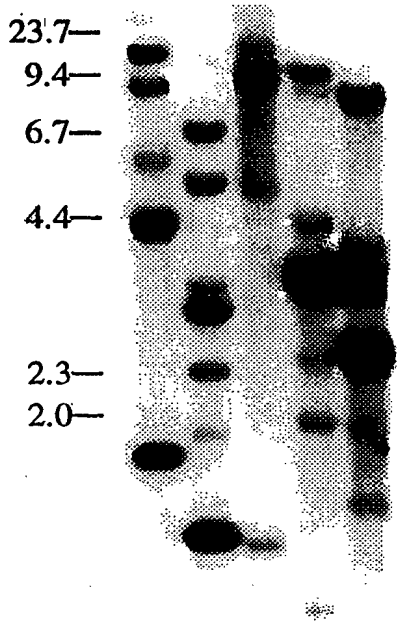
FIG. 5E

FIG. 6A



Tm - 8 °C

FIG. 6B



Tm - 33 °C

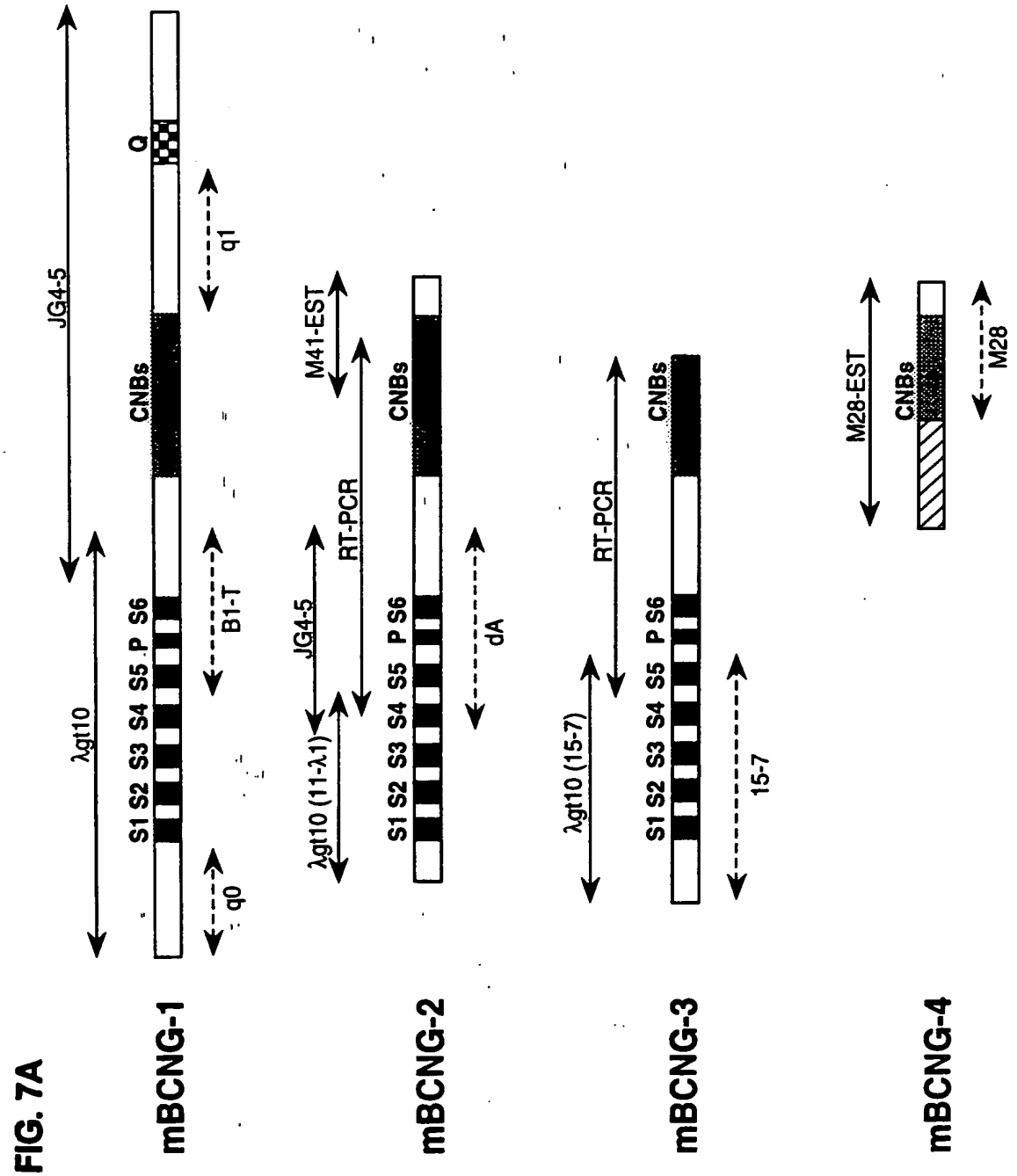


FIG. 7A

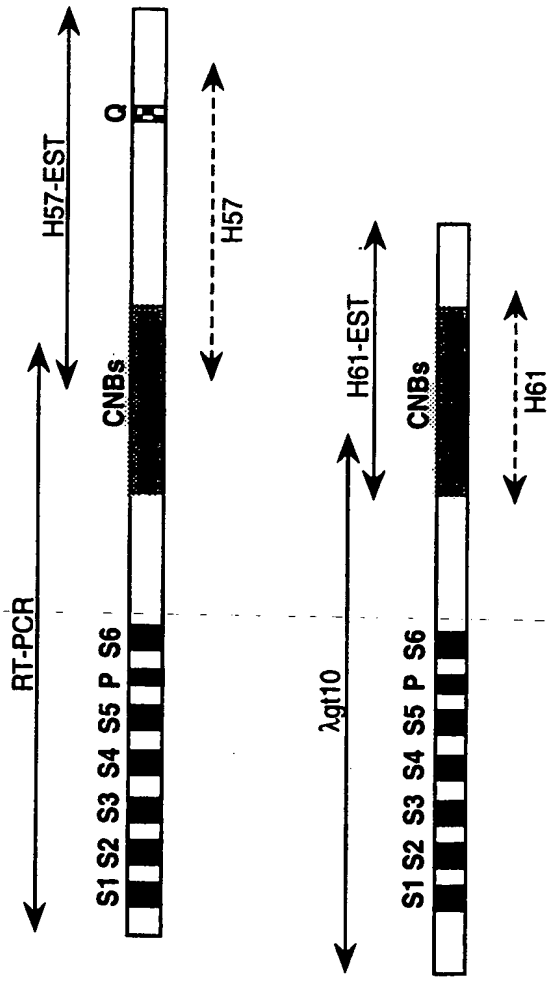


FIG. 7B

FIG. 7C

	mBCNG-2	mBCNG-3	hBCNG-1	hBCNG-2
mBCNG-1	84 %	86 %	100 %	84 %
mBCNG-2		89 %	84 %	98 %
mBCNG-3			86 %	89 %
hBCNG-1				84 %

FIG. 8A

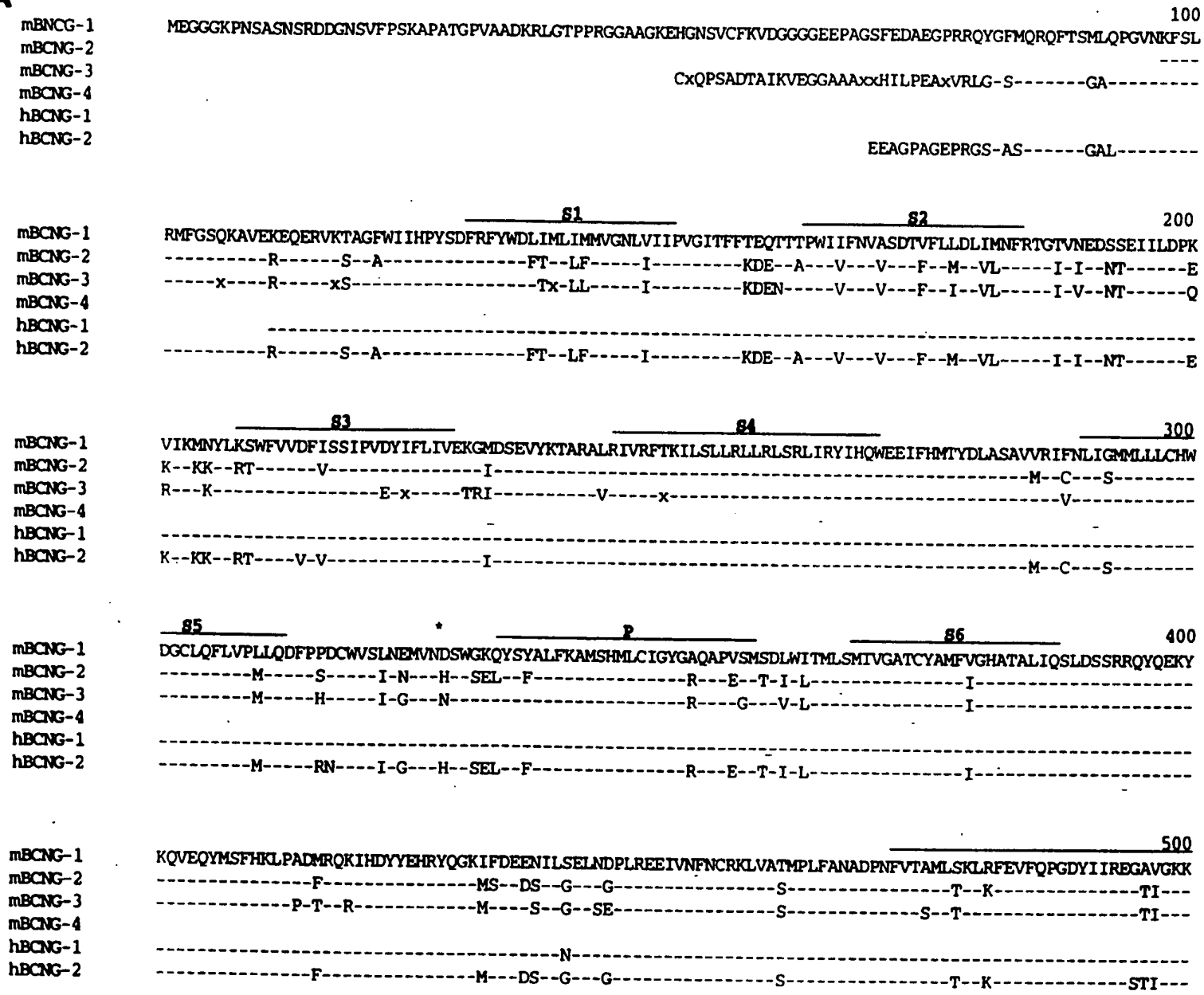


FIG. 8B

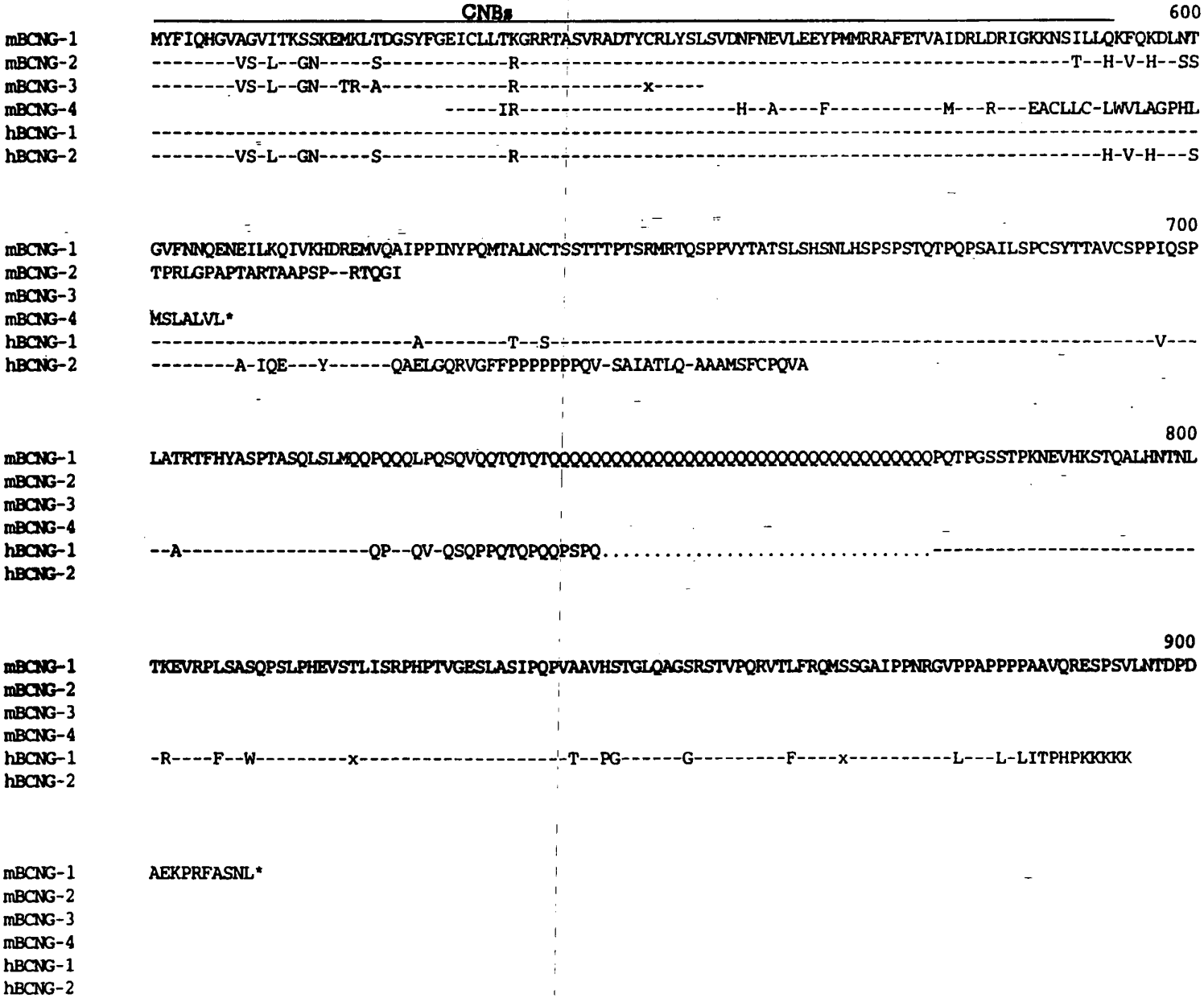


FIG. 9A

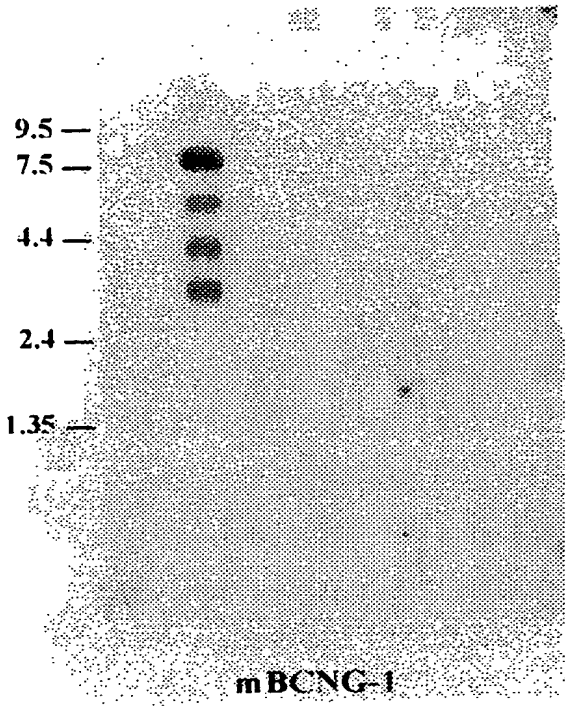


FIG. 9B

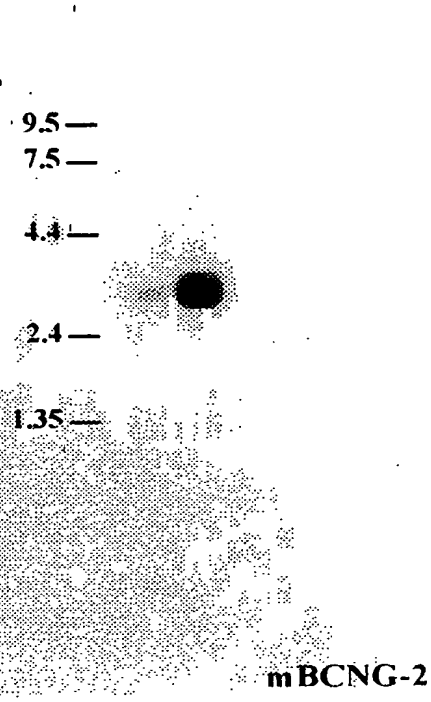


FIG. 9C

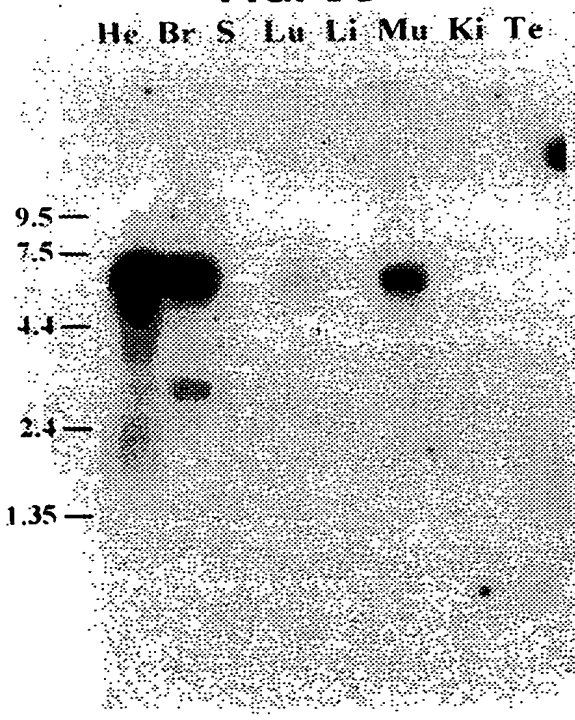


FIG. 9D

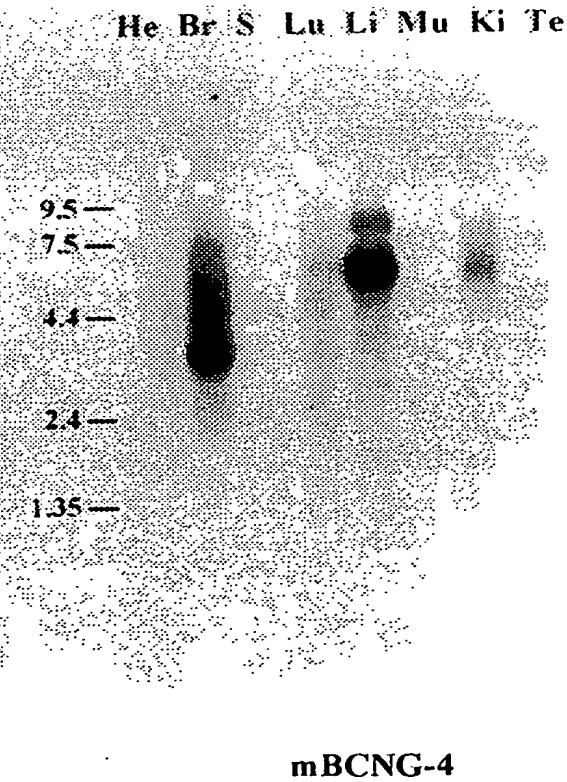
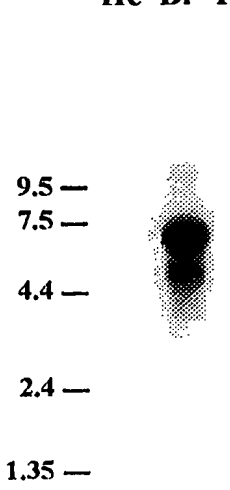
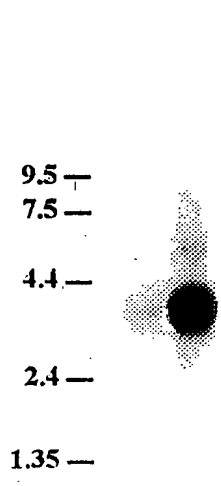


FIG. 10A

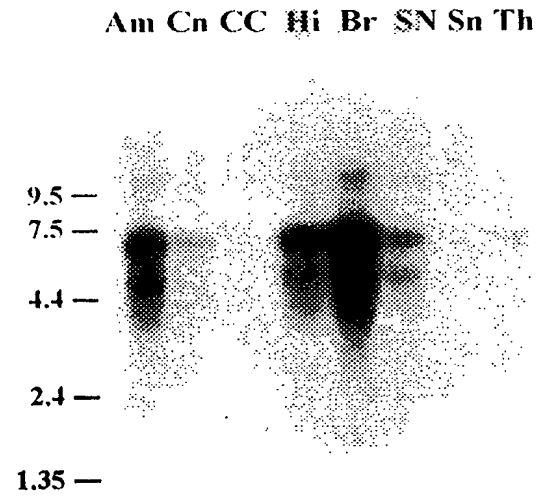


hBCNG-1

FIG. 10B

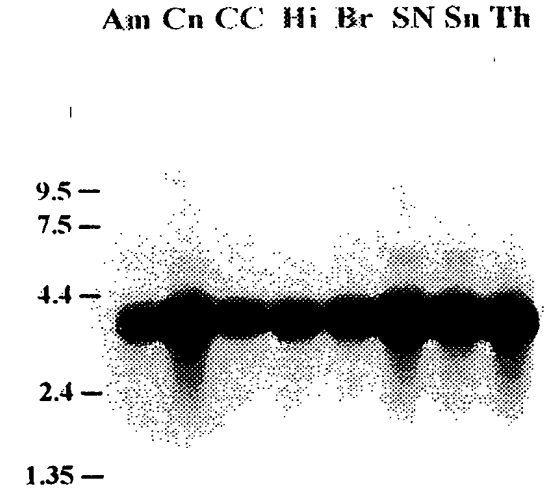


hBCNG-2



hBCNG-1

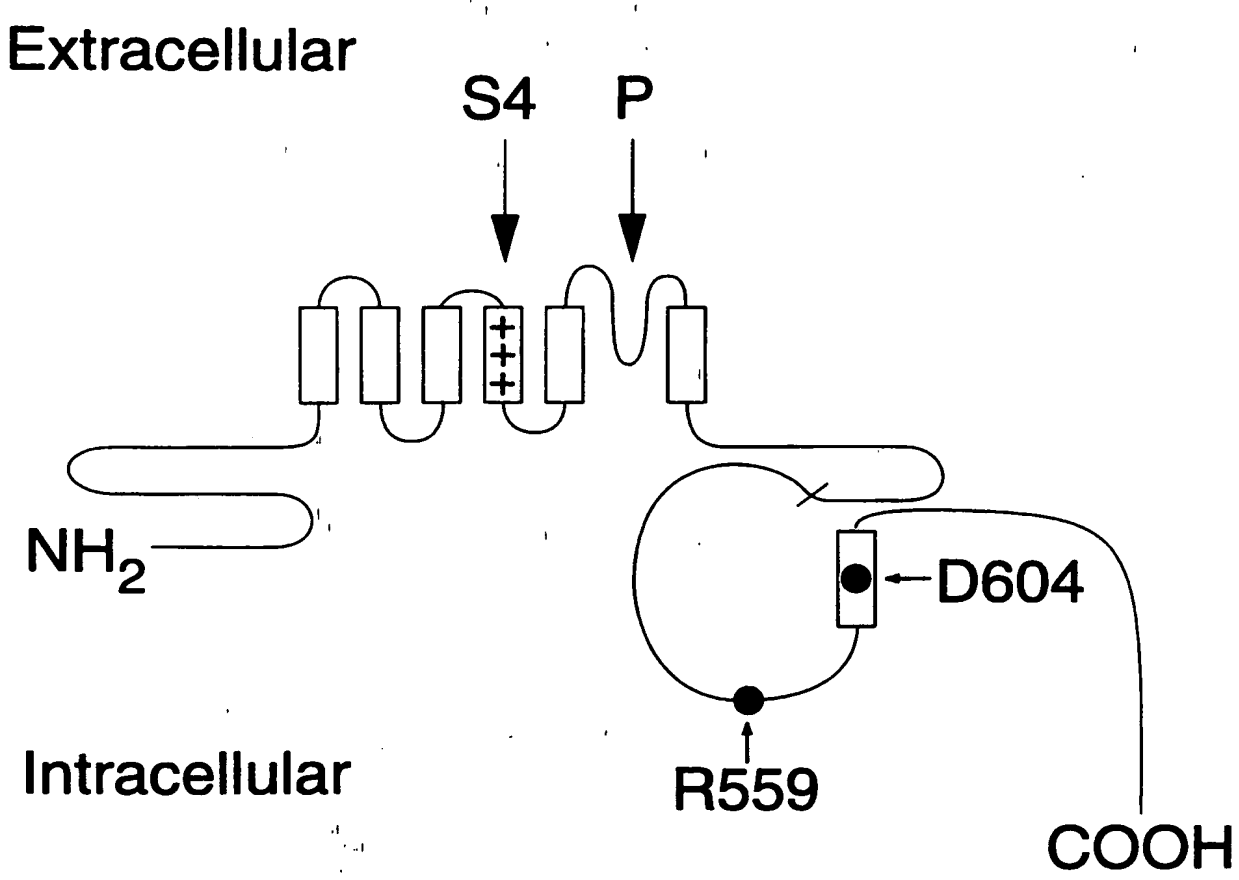
FIG. 10C



hBCNG-2

FIG. 10D

FIG. 11



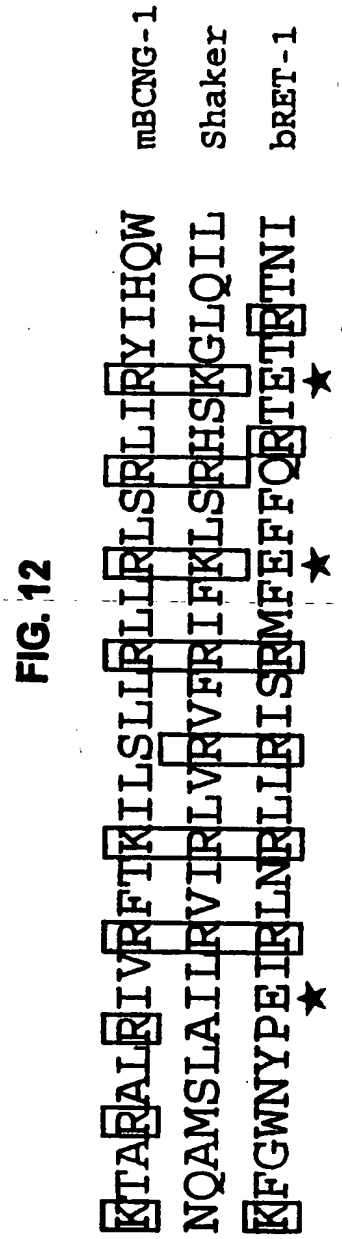
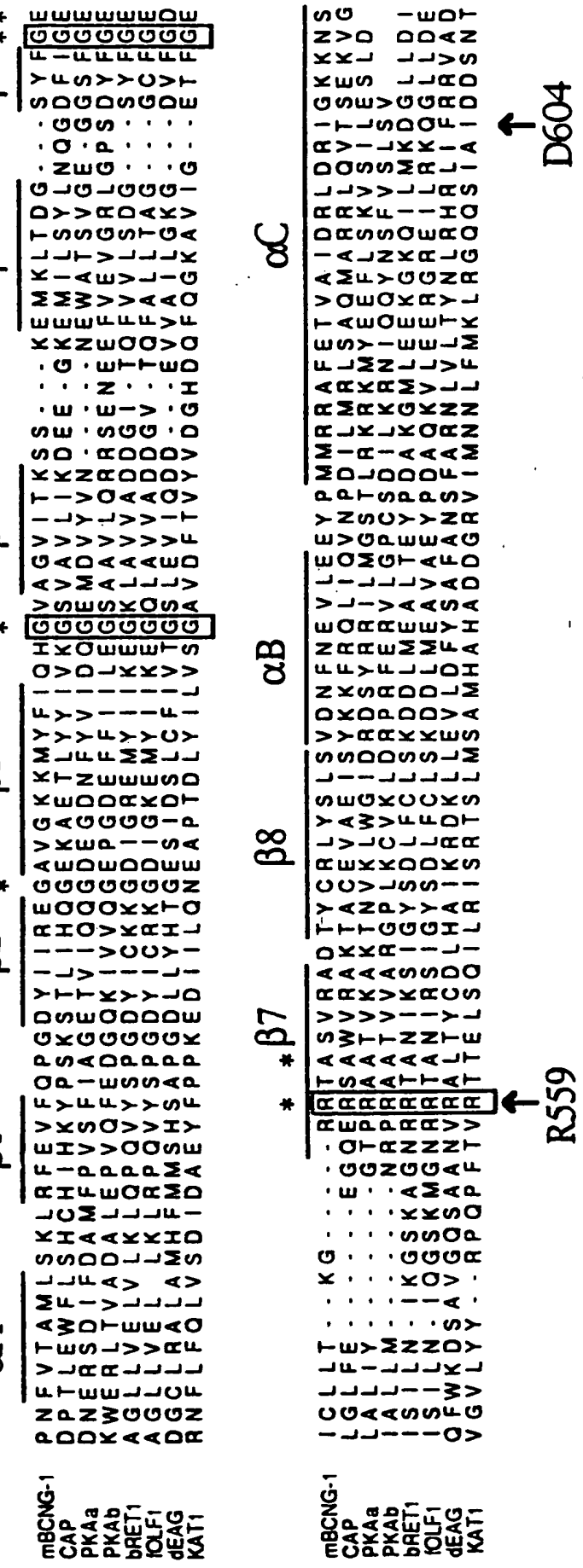


FIG. 12

FIG. 13A



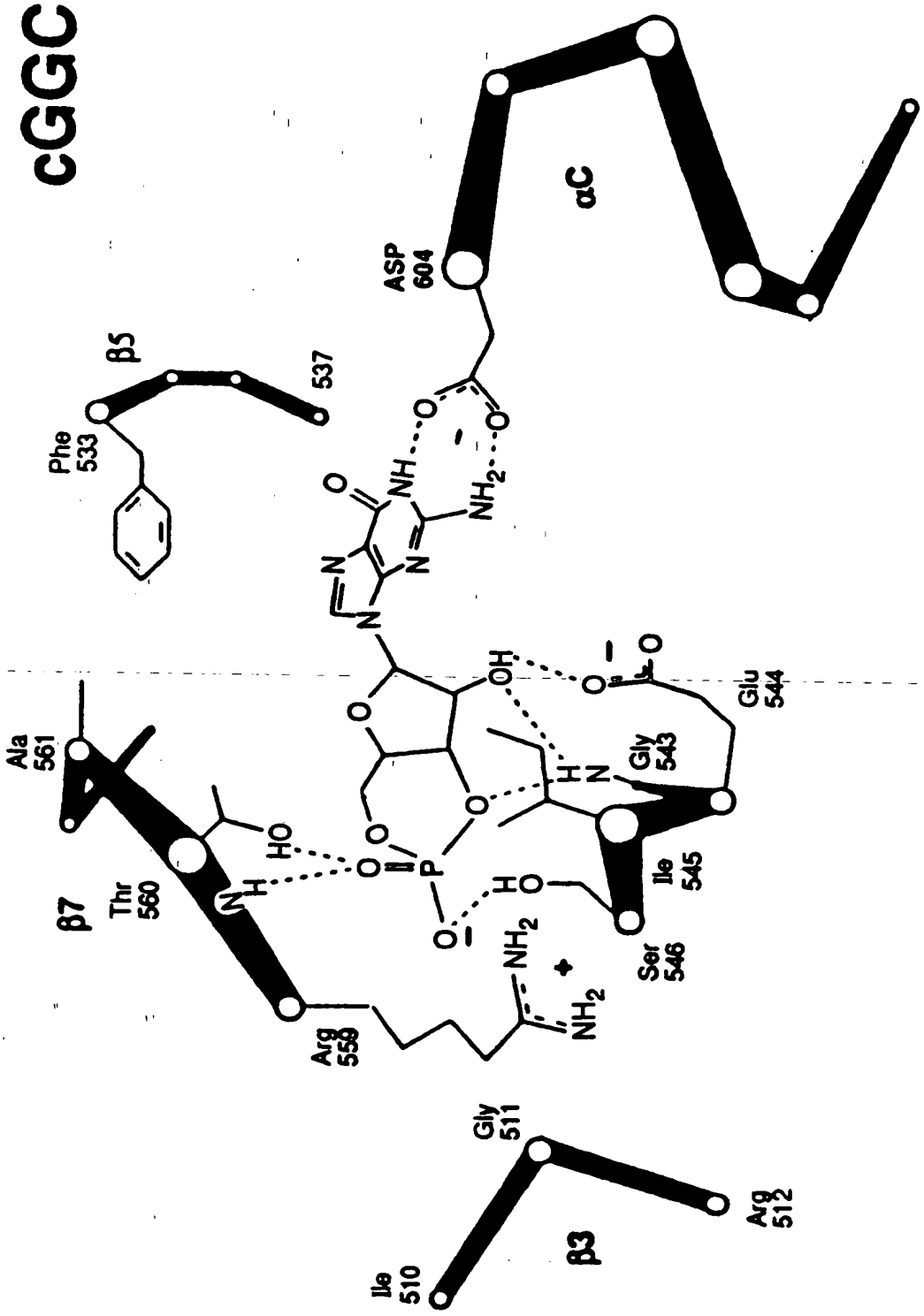
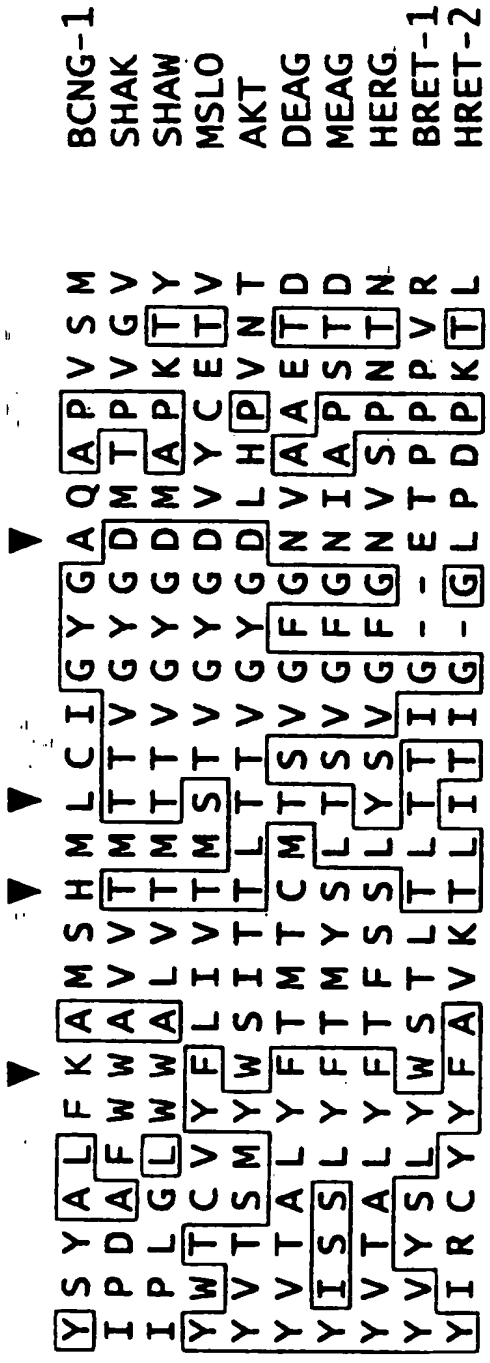


FIG. 13B

FIG. 14



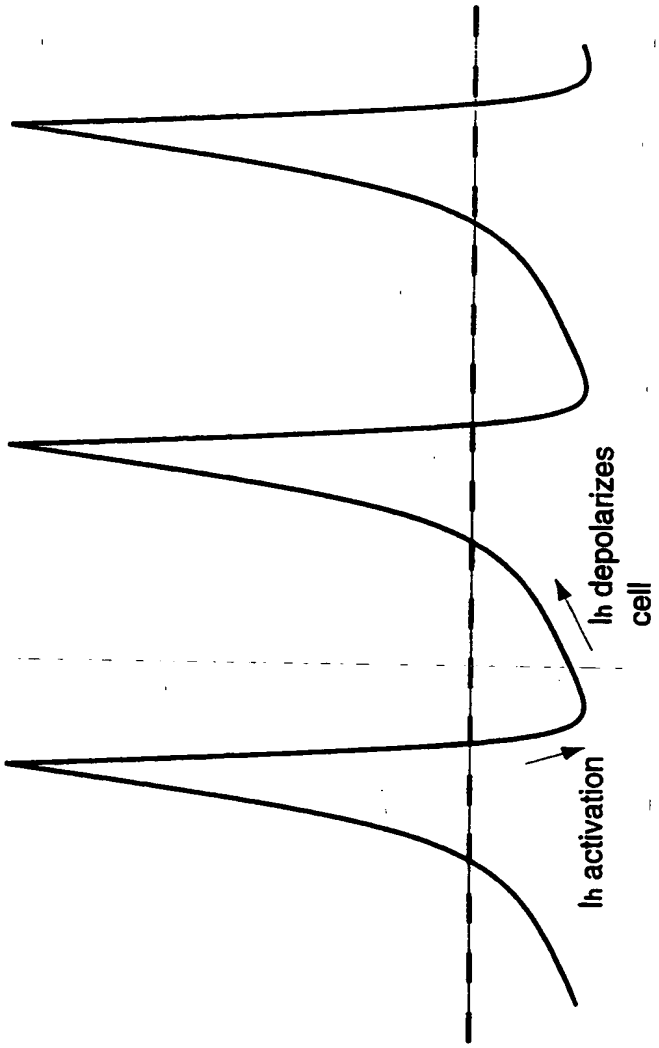
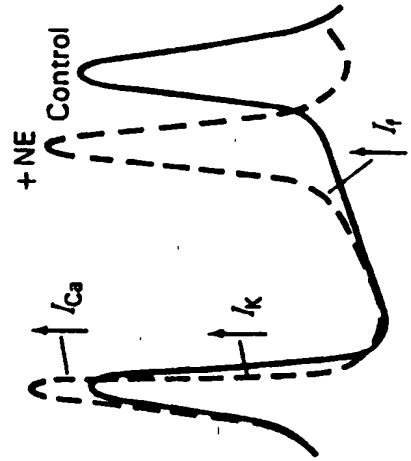


FIG. 15A



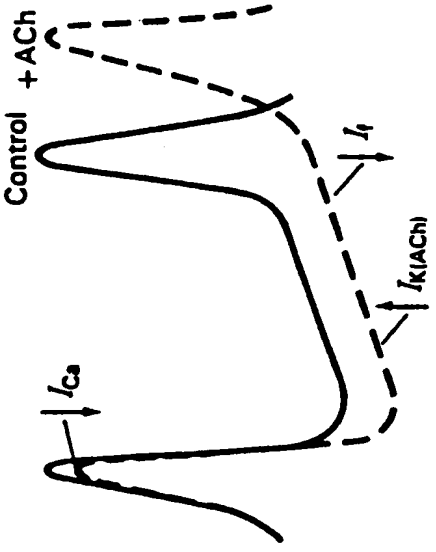


FIG. 15B

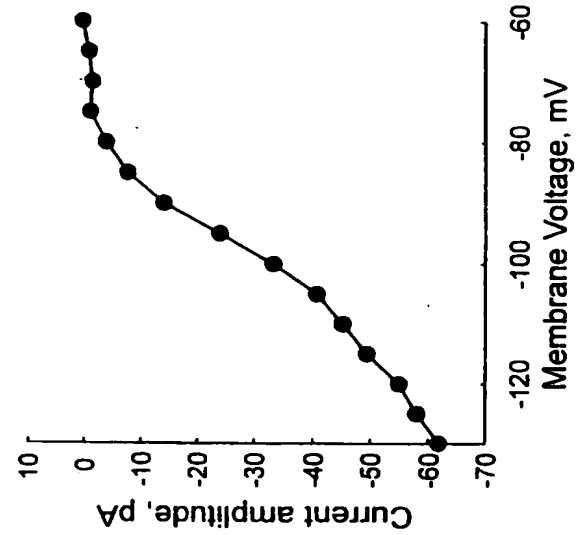


FIG. 16B

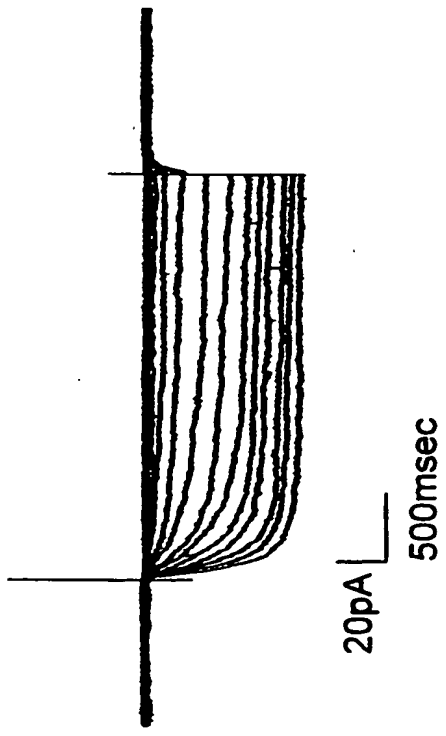


FIG. 16A

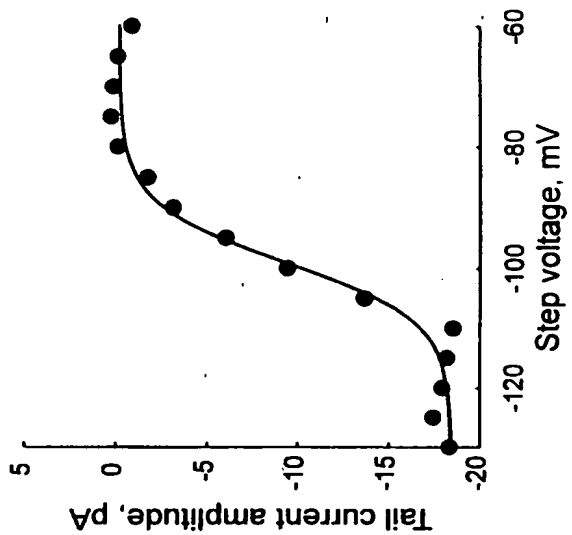


FIG. 16D

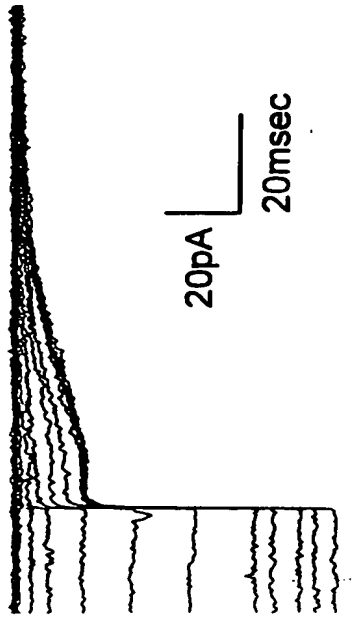


FIG. 16C

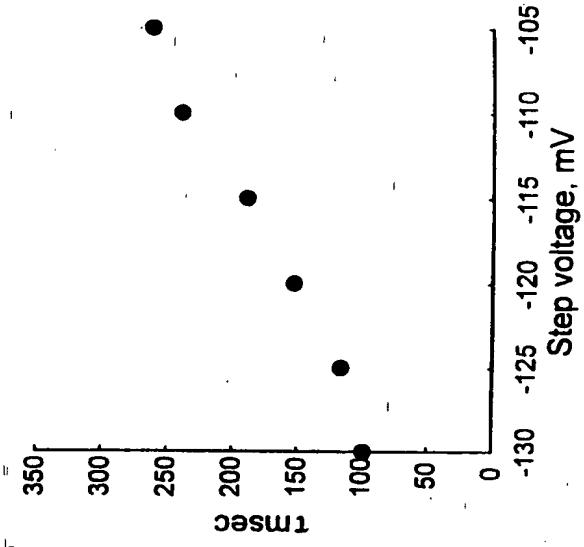


FIG. 16F

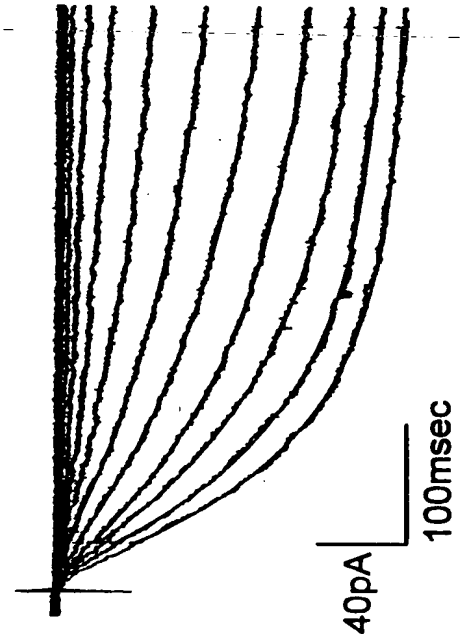


FIG. 16E

FIG. 17B

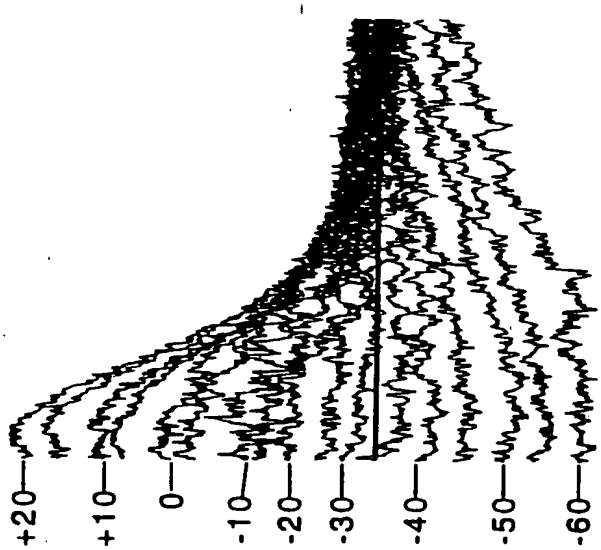


FIG. 17A

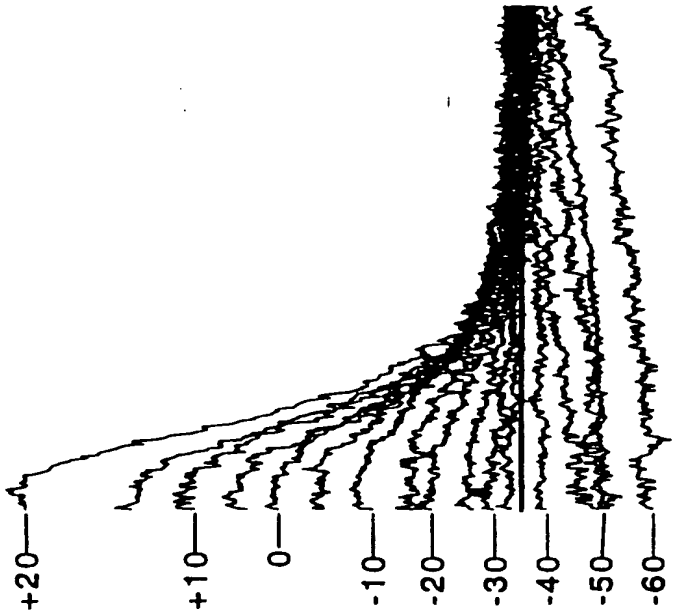


FIG. 17C

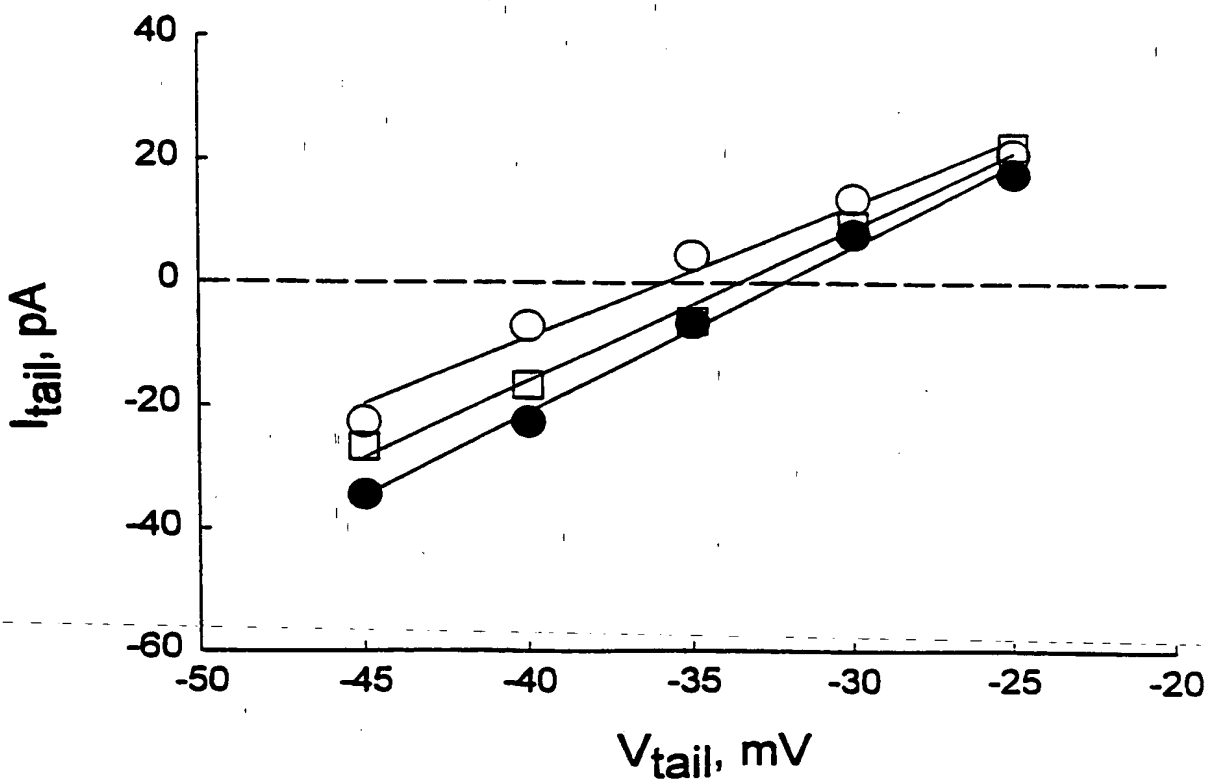
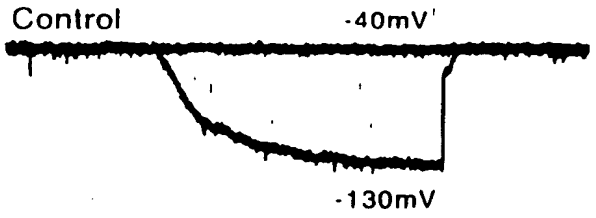
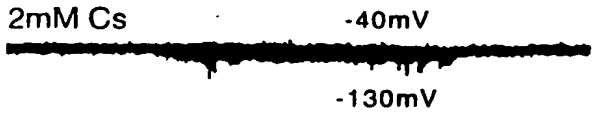


FIG. 18A





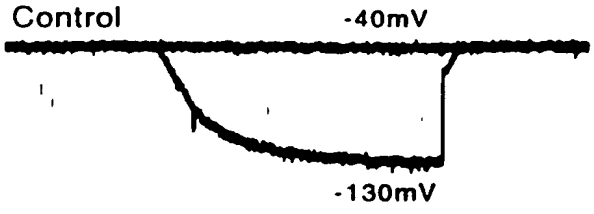
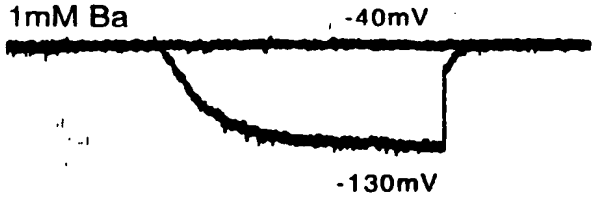
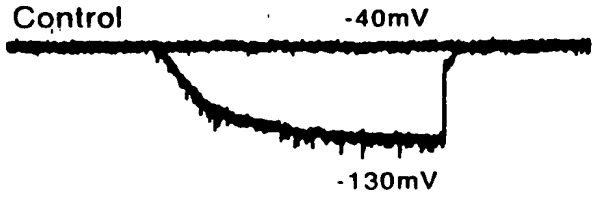


FIG. 18B





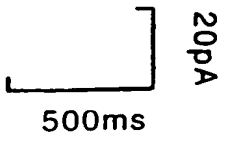
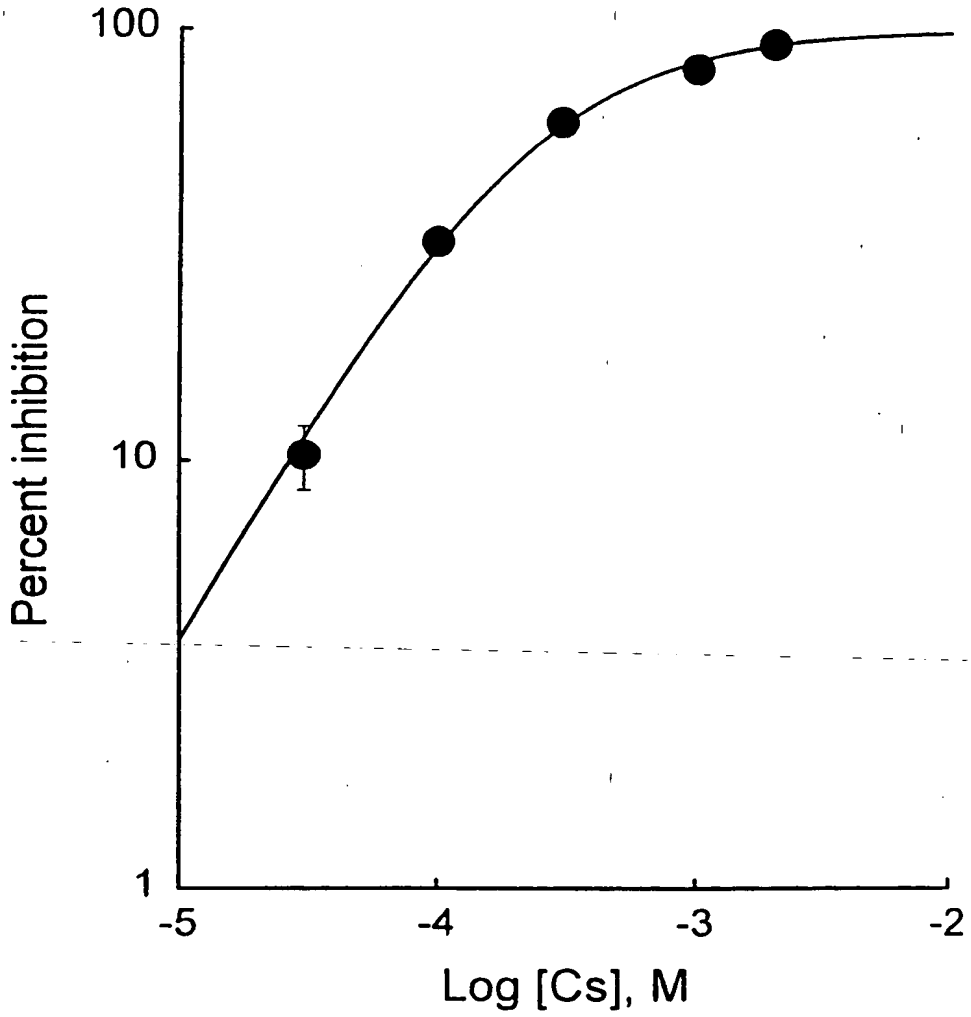


FIG. 18C



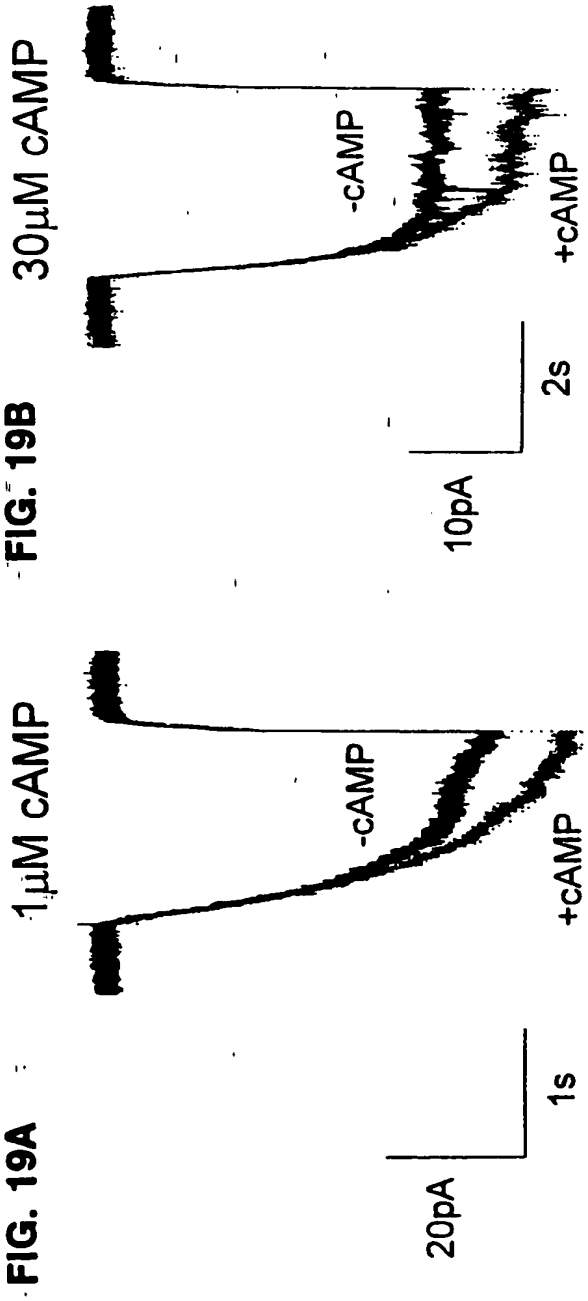


FIG. 19B

FIG. 19A

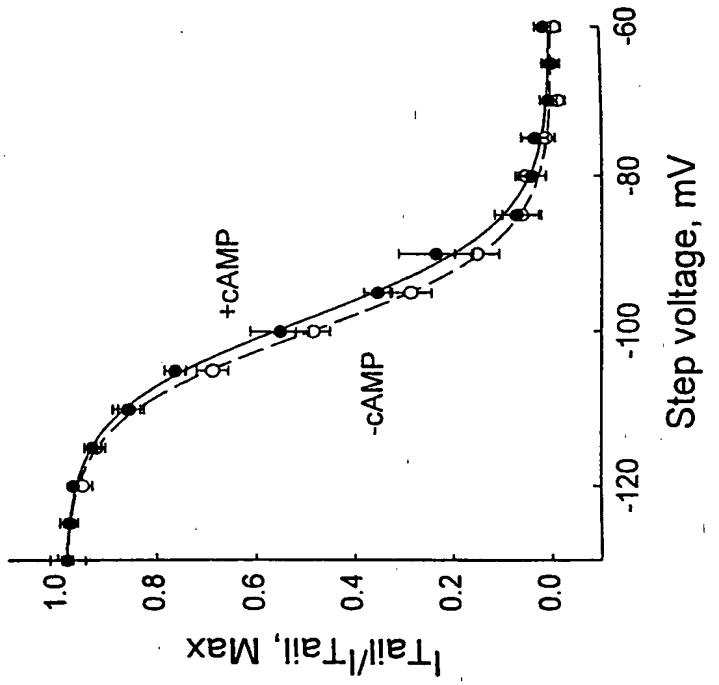


FIG. 19D

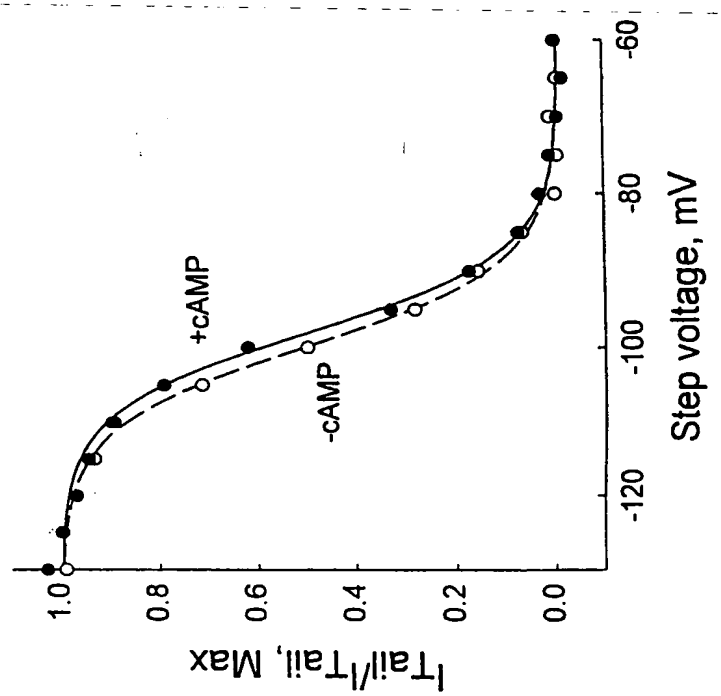


FIG. 19C

FIG. 20A

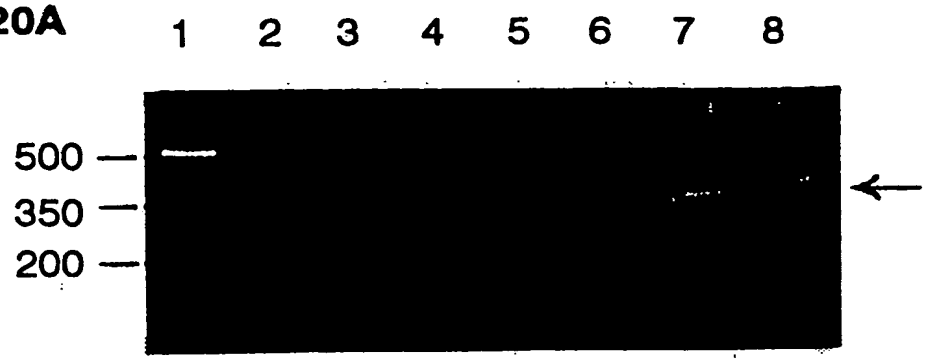


FIG. 20B

FIG. 20C

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kandel, Eric R
Siegelbaum, Steven A
Santoro, Bina
Tibbs, Gareth R
Bartsch, Dusan
Grant, Seth

(ii) TITLE OF INVENTION: BRAIN CYCLIC NUCLEOTIDE CHANNELS AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 34

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Cooper & Dunham, LLP
(B) STREET: 1185 Avenue of the Americas
(C) CITY: New York
(D) STATE: NY
(E) COUNTRY: USA
(F) ZIP: 10036

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: White, John P
(B) REGISTRATION NUMBER: 28,678
(C) REFERENCE/DOCKET NUMBER: 0575-54806

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 278-0400
(B) TELEFAX: (212) 391-0525

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2733 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGAAGGCG GCGGCAAACC CAACTCCGCG TCCAACAGCC GCGACGATGG CAACAGCGTC 60

TTCCCCTCCA AGGCGCCCGC GACGGGGCCG GTGGCGGCCG ACAAGCGCCT GGGGACCCCG 120

CCGAGGGGCG GCGCGGCCGG GAAGGAACAT GGCAACTCCG TGTGCTTCAA GGTGGACGGC 180

GGCGGAGGAG AGGAGCCGGC GGGCAGCTTC GAGGATGCCG AGGGGCCCCG GCGGCAGTAT 240

GGTTTCATGC AGAGGCAGTT CACCTCCATG CTGCAGCCTG GGGTCAACAA ATTCTCCCTC 300

CGCATGTTTG GGAGCCAGAA GGCGGTGGAG AAGGAGCAGG AAAGGGTTAA AACTGCAGGC 360

TTCTGGATTA TCCATCCGTA CAGTGACTTC AGGTTTTATT GGGATTTAAT CATGCTTATA 420

ATGATGGTTG GAAATTTGGT CATCATACCA GTTGGAATCA CGTTCTTCAC AGAGCAGACG 480

ACAACACCGT GGATTATTTT CAACGTGGCA TCCGATACTG TTTTCCTGTT GGACTTAATC 540

ATGAATTTTA GGACTGGGAC TGTCAATGAA GACAGCTCGG AAATCATCCT GGACCCTAAA 600

GTGATCAAGA TGAATTATTT AAAAAGCTGG TTTGTGGTGG ACTTCATCTC ATCGATCCCG 660

GTGGATTATA TCTTTCTCAT TGTAGAGAAA GGGATGGACT CAGAAGTTTA CAAGACAGCC 720

AGAGCACTTC GTATCGTGAG GTTTACAAAA ATTCTCAGTC TCTTGCGGTT ATTACGCCTT 780

TCAAGGTTAA TCAGATACAT ACACCAGTGG GAAGAGATAT TCCACATGAC CTATGACCTC 840

GCCAGTGCTG TGGTGAGGAT CTTCAACCTC ATTGGCATGA TGCTGCTTCT GTGCCACTGG 900

GATGGCTGTC TTCAGTTCCT GGTTCCCCTG CTGCAGGACT TCCCACCAGA TTGCTGGGTT 960

TCTCTGAATG AAATGGTTAA TGATTCCTGG GGAAAACAAT ATTCCTACGC ACTCTTCAAA 1020

GCTATGAGTC ACATGCTGTG CATTGGTTAT GGCGCCCAAG CCCCTGTCAG CATGTCTGAC 1080

CTCTGGATTA CCATGCTGAG CATGATTGTG GGCGCCACCT GCTACGCAAT GTTTGTTGGC 1140

CATGCCACAG CTTTGATCCA GTCTTTGGAC TCTTCAAGGA GGCAGTATCA AGAGAAGTAT 1200

AAGCAAGTAG AGCAATACAT GTCATTCCAC AAGTTACCAG CTGACATGCG CCAGAAGATA 1260

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ACTATGCCTC TTTTTGCTAA CGCCGATCCC AATTTCGTGA CGGCCATGCT GAGCAAGCTG 1440

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AGTGTCCGAG CTGATACCTA CTGTCGTCTT TACTCCCTTT CGGTGGACAA TTTCAATGAG 1680

GTCTTGGAGG AATATCCAAT GATGAGAAGA GCCTTTGAGA CAGTTGCTAT TGACCGACTC 1740

GATCGGATAG GCAAGAAAAA CTCTATTCTC CTGCAGAAGT TCCAGAAGGA TCTAAACACT 1800

GGTGTTTTCA ACAACCAGGA GAACGAGATC CTGAAGCAGA TCGTGAAGCA TGACCGAGAG 1860

ATGGTACAAG CTATCCCTCC AATCAACTAT CCTCAAATGA CAGCCCTCAA CTGCACATCT 1920

TCAACCACCA CCCCAACCTC CCGCATGAGG ACCCAATCTC CGCCAGTCTA CACCGCAACC 1980

AGCCTGTCTC ACAGCAATCT GCACTCACCC AGTCCCAGCA CACAGACGCC CCAACCCTCA 2040

GCCATCCTTT CACCCTGCTC CTATACCACA GCAGTCTGCA GTCCTCCTAT ACAGAGCCCC 2100

CTGGCCACAC GAACTTTCCA TTATGCCTCT CCCACTGCGT CCCAGCTGTC ACTCATGCAG 2160

CAGCCTCAGC AGCAACTACC GCAGTCCCAG GTACAGCAGA CTCAGACTCA GACTCAGCAG 2220

CAGCAGCAGC AACAGCAGCA GCAGCAGCAG CAGCAACAGC AACAACAGCA GCAGCAGCAG 2280

CAGCAGCAGC AGCAGCAGCA GCAGCAGCAG CAGCAGCAGC AGCAGCCACA GACACCTGGT 2340

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ACCAAAGAAG TCAGGCCCCT TTCCGCCTCG CAGCCTTCTC TGCCCCATGA GGTCTCCACT 2460

TTGATCTCCA GACCTCATCC CACTGTGGGC GAATCCCTGG CCTCTATCCC TCAACCCGTG 2520

GCAGCAGTCC ACAGCACTGG CCTTCAGGCA GGGAGCAGGA GCACAGTGCC ACAACGTGTC 2580

ACCTTGTTCC GACAGATGTC CTCGGGAGCC ATCCCCCCCA ACCGAGGAGT GCCTCCAGCA 2640

CCCCCTCCAC CAGCAGCTGT GCAGAGAGAG TCTCCCTCAG TCCTAAATAC AGACCCAGAT 2700

GCAGAAAAAC CCCGTTTTGC TTCGAATTTA TGA 2733

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 910 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Gly Gly Gly Lys Pro Asn Ser Ala Ser Asn Ser Arg Asp Asp
1 5 10 15

Gly Asn Ser Val Phe Pro Ser Lys Ala Pro Ala Thr Gly Pro Val Ala
20 25 30

Ala Asp Lys Arg Leu Gly Thr Pro Pro Arg Gly Gly Ala Ala Gly Lys
35 40 45

Glu His Gly Asn Ser Val Cys Phe Lys Val Asp Gly Gly Gly Gly Glu
50 55 60

Glu Pro Ala Gly Ser Phe Glu Asp Ala Glu Gly Pro Arg Arg Gln Tyr
65 70 75 80

Gly Phe Met Gln Arg Gln Phe Thr Ser Met Leu Gln Pro Gly Val Asn
85 90 95

```
Lys Phe Ser Leu Arg Met Phe Gly Ser Gln Lys Ala Val Glu Lys Glu
            100                 105                 110

Gln Glu Arg Val Lys Thr Ala Gly Phe Trp Ile Ile His Pro Tyr Ser
        115                 120                 125

Asp Phe Arg Phe Tyr Trp Asp Leu Ile Met Leu Ile Met Met Val Gly
    130                 135                 140

Asn Leu Val Ile Ile Pro Val Gly Ile Thr Phe Phe Thr Glu Gln Thr
145                 150                 155                 160

Thr Thr Pro Trp Ile Ile Phe Asn Val Ala Ser Asp Thr Val Phe Leu
                165                 170                 175

Leu Asp Leu Ile Met Asn Phe Arg Thr Gly Thr Val Asn Glu Asp Ser
            180                 185                 190

Ser Glu Ile Ile Leu Asp Pro Lys Val Ile Lys Met Asn Tyr Leu Lys
        195                 200                 205

Ser Trp Phe Val Val Asp Phe Ile Ser Ser Ile Pro Val Asp Tyr Ile
    210                 215                 220

Phe Leu Ile Val Glu Lys Gly Met Asp Ser Glu Val Tyr Lys Thr Ala
225                 230                 235                 240

Arg Ala Leu Arg Ile Val Arg Phe Thr Lys Ile Leu Ser Leu Leu Arg
                245                 250                 255

Leu Leu Arg Leu Ser Arg Leu Ile Arg Tyr Ile His Gln Trp Glu Glu
            260                 265                 270

Ile Phe His Met Thr Tyr Asp Leu Ala Ser Ala Val Val Arg Ile Phe
        275                 280                 285

Asn Leu Ile Gly Met Met Leu Leu Leu Cys His Trp Asp Gly Cys Leu
    290                 295                 300

Gln Phe Leu Val Pro Leu Leu Gln Asp Phe Pro Pro Asp Cys Trp Val
305                 310                 315                 320

Ser Leu Asn Glu Met Val Asn Asp Ser Trp Gly Lys Gln Tyr Ser Tyr
                325                 330                 335

Ala Leu Phe Lys Ala Met Ser His Met Leu Cys Ile Gly Tyr Gly Ala
            340                 345                 350

Gln Ala Pro Val Ser Met Ser Asp Leu Trp Ile Thr Met Leu Ser Met
        355                 360                 365

Ile Val Gly Ala Thr Cys Tyr Ala Met Phe Val Gly His Ala Thr Ala
    370                 375                 380

Leu Ile Gln Ser Leu Asp Ser Ser Arg Arg Gln Tyr Gln Glu Lys Tyr
385                 390                 395                 400

Lys Gln Val Glu Gln Tyr Met Ser Phe His Lys Leu Pro Ala Asp Met
                405                 410                 415

Arg Gln Lys Ile His Asp Tyr Tyr Glu His Arg Tyr Gln Gly Lys Ile
            420                 425                 430
```

Phe Asp Glu Glu Asn Ile Leu Ser Glu Leu Asn Asp Pro Leu Arg Glu
435 440 445

Glu Ile Val Asn Phe Asn Cys Arg Lys Leu Val Ala Thr Met Pro Leu
450 455 460

Phe Ala Asn Ala Asp Pro Asn Phe Val Thr Ala Met Leu Ser Lys Leu
465 470 475 480

Arg Phe Glu Val Phe Gln Pro Gly Asp Tyr Ile Ile Arg Glu Gly Ala
485 490 495

Val Gly Lys Lys Met Tyr Phe Ile Gln His Gly Val Ala Gly Val Ile
500 505 510

Thr Lys Ser Ser Lys Glu Met Lys Leu Thr Asp Gly Ser Tyr Phe Gly
515 520 525

Glu Ile Cys Leu Leu Thr Lys Gly Arg Arg Thr Ala Ser Val Arg Ala
530 535 540

Asp Thr Tyr Cys Arg Leu Tyr Ser Leu Ser Val Asp Asn Phe Asn Glu
545 550 555 560

Val Leu Glu Glu Tyr Pro Met Met Arg Arg Ala Phe Glu Thr Val Ala
565 570 575

Ile Asp Arg Leu Asp Arg Ile Gly Lys Lys Asn Ser Ile Leu Leu Gln
580 585 590

Lys Phe Gln Lys Asp Leu Asn Thr Gly Val Phe Asn Asn Gln Glu Asn
595 600 605

Glu Ile Leu Lys Gln Ile Val Lys His Asp Arg Glu Met Val Gln Ala
610 615 620

Ile Pro Pro Ile Asn Tyr Pro Gln Met Thr Ala Leu Asn Cys Thr Ser
625 630 635 640

Ser Thr Thr Thr Pro Thr Ser Arg Met Arg Thr Gln Ser Pro Pro Val
645 650 655

Tyr Thr Ala Thr Ser Leu Ser His Ser Asn Leu His Ser Pro Ser Pro
660 665 670

Ser Thr Gln Thr Pro Gln Pro Ser Ala Ile Leu Ser Pro Cys Ser Tyr
675 680 685

Thr Thr Ala Val Cys Ser Pro Pro Ile Gln Ser Pro Leu Ala Thr Arg
690 695 700

Thr Phe His Tyr Ala Ser Pro Thr Ala Ser Gln Leu Ser Leu Met Gln
705 710 715 720

Gln Pro Gln Gln Gln Leu Pro Gln Ser Gln Val Gln Gln Thr Gln Thr
725 730 735

Gln Thr Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
740 745 750

Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
755 760 765

Gln Gln Gln Gln Gln Gln Pro Gln Thr Pro Gly Ser Ser Thr Pro
770 775 780

Lys Asn Glu Val His Lys Ser Thr Gln Ala Leu His Asn Thr Asn Leu
785 790 795 800

Thr Lys Glu Val Arg Pro Leu Ser Ala Ser Gln Pro Ser Leu Pro His
805 810 815

Glu Val Ser Thr Leu Ile Ser Arg Pro His Pro Thr Val Gly Glu Ser
820 825 830

Leu Ala Ser Ile Pro Gln Pro Val Ala Ala Val His Ser Thr Gly Leu
835 840 845

Gln Ala Gly Ser Arg Ser Thr Val Pro Gln Arg Val Thr Leu Phe Arg
850 855 860

Gln Met Ser Ser Gly Ala Ile Pro Pro Asn Arg Gly Val Pro Pro Ala
865 870 875 880

Pro Pro Pro Pro Ala Ala Val Gln Arg Glu Ser Pro Ser Val Leu Asn
885 890 895

Thr Asp Pro Asp Ala Glu Lys Pro Arg Phe Ala Ser Asn Leu
900 905 910

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2263 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAGGAGCAGG AAAGGGTTAA AACTGCAGGC TTCTGGATTA TCCACCCTTA CAGTGATTTC 60

AGGTTTTACT GGGATTTAAT AATGCTCATA ATGATGGTTG GAAATCTAGT CATCATACCA 120

GTTGGAATCA CATTCTTTAC AGAGCAAACA ACAACACCAT GGATTATTTT CAATGTGGCA 180

TCAGATACAG TTTTCCTATT GGACCTGATC ATGAATTTTA GGACTGGGAC TGTCAATGAA 240

GACAGTTCTG AAATCATCCT GGACCCCAAA GTGATCAAGA TGAATTATTT AAAAAGCTGG 300

TTTGTGGTTG ACTTCATCTC ATCCATCCCA GTGGATTATA TCTTTCTTAT TGTAGAAAAA 360

GGAATGGATT CTGAAGTTTA CAAGACAGCC AGGGCCCTTC GCATTGTGAG GTTTACAAAA 420

ATTCTCAGTC TCTTGCGTTT ATTACGACTT TCAAGGTTAA TTAGATACAT ACATCAATGG 480

GAAGAGATAT TCCACATGAC ATATGATCTC GCCAGTGCAG TGGTGAGAAT TTTTAATCTC 540

ATCGGCATGA TGCTGCTCCT GTGCCACTGG GATGGTTGTC TTCAGTTCTT AGTACCACTA 600

CTGCAGGACT TCCCACCAGA TTGCTGGGTG TCTTTAAATG AAATGGTTAA TGATTCTTGG 660

GGAAAGCAGT ATTCATACGC ACTCTTCAAA GCTATGAGTC ACATGCTGTG CATTGGGTAT 720

GGAGCCCAAG CCCCAGTCAG CATGTCTGAC CTCTGGATTA CCATGCTGAG CATGATCGTC 780

GGGGCCACCT GCTATGCCAT GTTTGTCGGC CATGCCACCG CTTTAATCCA GTCTCTGGAT 840

TCTTCGAGGC GGCAGTATCA AGAGAAGTAT AAGCAAGTGG AACAATACAT GTCATTCCAT 900

AAGTTACCAG CTGATATGCG TCAGAAGATA CATGATTACT ATGAACACAG ATACCAAGGC 960

AAAATCTTTG ATGAGGAAAA TATTCTCAAT GAACTCAATG ATCCTCTGAG AGAGGAGATA 1020

GTCAACTTCA ACTGTCGGAA ACTGGTGGCT ACAATGCCTT TATTTGCTAA TGCGGATCCT 1080

AATTTTGTGA CTGCCATGCT GAGCAAGTTG AGATTTGAGG TGTTTCAACC TGGAGATTAT 1140

ATCATACGAG AAGGAGCCGT GGGTAAAAAA ATGTATTTCA TTCAACACGG TGTTGCTGGT 1200

GTCATTACAA AATCCAGTAA AGAAATGAAG CTGACAGATG GCTCTTACTT TGGAGAGATT 1260

TGCCTGCTGA CCAAAGGACG TCGTACTGCC AGTGTTCGAG CTGATACATA TTGTCGTCTT 1320

TACTCACTTT CCGTGGACAA TTTCAACGAG GTCCTGGAGG AATATCCAAT GATGAGGAGA 1380

GCCTTTGAGA CAGTTGCCAT TGACCGACTA GATCGAATAG GAAAGAAAAA TTCAATTCTT 1440

CTGCAAAAGT TCCAGAAGGA TCTGAACACT GGTGTTTTCA ACAATCAGGA GAACGAAATC 1500

CTCAAGCAGA TTGTGAAACA TGACAGGGAG ATGGTGCAGG CAATCGCTCC CATCAATTAT 1560

CCTCAAATGA CAACCCTGAA TTCCACATCG TCTACTACGA CCCCGACCTC CCGCATGAGG 1620

ACACAATCTC CACCGGTGTA CACAGCGACC AGCCTGTCTC ACAGCAACCT GCACTCCCCC 1680

AGTCCCAGCA CACAGACCCC CCAGCCATCA GCCATCCTGT CACCCTGCTC CTACACCACC 1740

GCGGTCTGCA GCCCTCCTGT ACAGAGCCCT CTGGCCGCTC GAACTTTCCA CTATGCCTCC 1800

CCCACCGCCT CCCAGCTGTC ACTCATGCAA CAGCAGCCGC AGCAGCAGGT ACAGCAGTCC 1860

CAGCCGCCGC AGACTCAGCC ACAGCAGCCG TCCCCGCAGC CACAGACACC TGGCAGCTCC 1920

ACGCCGAAAA ATGAAGTGCA CAAGAGCACG CAGGCGCTTC ACAACACCAA CCTGACCCGG 1980

GAAGTCAGGC CATTTTCCGC CTGGCAGCCT CGCTGCCCCA TGAGGTGTCC ATTTTGATTT 2040

CCAGACCTCA TCCCACTGTG GGGGAGTCCC TGGCCTCCAT CCCTCAACCC GTGACGGCGG 2100

TCCCCGGAAC GGGCCTTCAG GCAGGGGGCA GGAGCACTGT CCCGCAGCGC GTCACCTTTT 2160

TCCGACAGAT GTGTCGGGAG CCATCCCCCC GAACCGAGGA GTCCTTCCAG CACCCCTTCC 2220

ACTTATCACA CCCCATCCTA AAAAAAAAAA AAAAAAAAAA AAA 2263

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 755 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Glu Gln Glu Arg Val Lys Thr Ala Gly Phe Trp Ile Ile His Pro
1 5 10 15

Tyr Ser Asp Phe Arg Phe Tyr Trp Asp Leu Ile Met Leu Ile Met Met
20 25 30

Val Gly Asn Leu Val Ile Ile Pro Val Gly Ile Thr Phe Phe Thr Glu
35 40 45

Gln Thr Thr Thr Pro Trp Ile Ile Phe Asn Val Ala Ser Asp Thr Val
50 55 60

Phe Leu Leu Asp Leu Ile Met Asn Phe Arg Thr Gly Thr Val Asn Glu
65 70 75 80

Asp Ser Ser Glu Ile Ile Leu Asp Pro Lys Val Ile Lys Met Asn Tyr
85 90 95

Leu Lys Ser Trp Phe Val Val Asp Phe Ile Ser Ser Ile Pro Val Asp
100 105 110

Tyr Ile Phe Leu Ile Val Glu Lys Gly Met Asp Ser Glu Val Tyr Lys
115 120 125

Thr Ala Arg Ala Leu Arg Ile Val Arg Phe Thr Lys Ile Leu Ser Leu
130 135 140

Leu Arg Leu Leu Arg Leu Ser Arg Leu Ile Arg Tyr Ile His Gln Trp
145 150 155 160

Glu Glu Ile Phe His Met Thr Tyr Asp Leu Ala Ser Ala Val Val Arg
165 170 175

Ile Phe Asn Leu Ile Gly Met Met Leu Leu Leu Cys His Trp Asp Gly
180 185 190

Cys Leu Gln Phe Leu Val Pro Leu Leu Gln Asp Phe Pro Pro Asp Cys
195 200 205

Trp Val Ser Leu Asn Glu Met Val Asn Asp Ser Trp Gly Lys Gln Tyr
210 215 220

Ser Tyr Ala Leu Phe Lys Ala Met Ser His Met Leu Cys Ile Gly Tyr
225 230 235 240

Gly Ala Gln Ala Pro Val Ser Met Ser Asp Leu Trp Ile Thr Met Leu
245 250 255

Ser Met Ile Val Gly Ala Thr Cys Tyr Ala Met Phe Val Gly His Ala
260 265 270

Thr Ala Leu Ile Gln Ser Leu Asp Ser Ser Arg Arg Gln Tyr Gln Glu
275 280 285

Lys Tyr Lys Gln Val Glu Gln Tyr Met Ser Phe His Lys Leu Pro Ala
290 295 300

Asp Met Arg Gln Lys Ile His Asp Tyr Tyr Glu His Arg Tyr Gln Gly

305 310 315 320

Lys Ile Phe Asp Glu Glu Asn Ile Leu Asn Glu Leu Asn Asp Pro Leu
325 330 335

Arg Glu Glu Ile Val Asn Phe Asn Cys Arg Lys Leu Val Ala Thr Met
340 345 350

Pro Leu Phe Ala Asn Ala Asp Pro Asn Phe Val Thr Ala Met Leu Ser
355 360 365

Lys Leu Arg Phe Glu Val Phe Gln Pro Gly Asp Tyr Ile Ile Arg Glu
370 375 380

Gly Ala Val Gly Lys Lys Met Tyr Phe Ile Gln His Gly Val Ala Gly
385 390 395 400

Val Ile Thr Lys Ser Ser Lys Glu Met Lys Leu Thr Asp Gly Ser Tyr
405 410 415

Phe Gly Glu Ile Cys Leu Leu Thr Lys Gly Arg Arg Thr Ala Ser Val
420 425 430

Arg Ala Asp Thr Tyr Cys Arg Leu Tyr Ser Leu Ser Val Asp Asn Phe
435 440 445

Asn Glu Val Leu Glu Glu Tyr Pro Met Met Arg Arg Ala Phe Glu Thr
450 455 460

Val Ala Ile Asp Arg Leu Asp Arg Ile Gly Lys Lys Asn Ser Ile Leu
465 470 475 480

Leu Gln Lys Phe Gln Lys Asp Leu Asn Thr Gly Val Phe Asn Asn Gln
485 490 495

Glu Asn Glu Ile Leu Lys Gln Ile Val Lys His Asp Arg Glu Met Val
500 505 510

Gln Ala Ile Ala Pro Ile Asn Tyr Pro Gln Met Thr Thr Leu Asn Ser
515 520 525

Thr Ser Ser Thr Thr Thr Pro Thr Ser Arg Met Arg Thr Gln Ser Pro
530 535 540

Pro Val Tyr Thr Ala Thr Ser Leu Ser His Ser Asn Leu His Ser Pro
545 550 555 560

Ser Pro Ser Thr Gln Thr Pro Gln Pro Ser Ala Ile Leu Ser Pro Cys
565 570 575

Ser Tyr Thr Thr Ala Val Cys Ser Pro Pro Val Gln Ser Pro Leu Ala
580 585 590

Ala Arg Thr Phe His Tyr Ala Ser Pro Thr Ala Ser Gln Leu Ser Leu
595 600 605

Met Gln Gln Gln Pro Gln Gln Gln Val Gln Gln Ser Gln Pro Pro Gln
610 615 620

Thr Gln Pro Gln Gln Pro Ser Pro Gln Pro Gln Thr Pro Gly Ser Ser
625 630 635 640

Thr Pro Lys Asn Glu Val His Lys Ser Thr Gln Ala Leu His Asn Thr

645 650 655

Asn Leu Thr Arg Glu Val Arg Pro Phe Ser Ala Trp Gln Pro Ser Leu
660 665 670

Pro His Glu Val Ser Ile Leu Ile Ser Arg Pro His Pro Thr Val Gly
675 680 685

Glu Ser Leu Ala Ser Ile Pro Gln Pro Val Thr Ala Val Pro Gly Thr
690 695 700

Gly Leu Gln Ala Gly Gly Arg Ser Thr Val Pro Gln Arg Val Thr Phe
705 710 715 720

Phe Arg Gln Met Xaa Ser Gly Ala Ile Pro Pro Asn Arg Gly Val Leu
725 730 735

Pro Ala Pro Leu Pro Leu Ile Thr Pro His Pro Lys Lys Lys Lys Lys
740 745 750

Lys Lys Lys
755

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1584 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAGTTCTCCC TGCGGATGTT CGGCAGCCAG AAGGCCGTGG AGCGCGAGCA GGAACGCGTG 60

AAGTCGGCGG GGGCCTGGAT CATCCACCCC TACAGCGACT TCAGGTTCTA CTGGGACTTC 120

ACCATGCTGT TGTTCATGGT GGGAAATCTC ATTATCATTC CCGTGGGCAT CACTTTCTTC 180

AAGGACGAGA CCACCGCGCC CTGGATCGTC TTCAACGTGG TCTCGGACAC TTTCTTCCTC 240

ATGGACTTGG TGTTGAACTT CCGCACCGGC ATTGTTATTG AGGACAACAC GGAGATCATC 300

CTGGACCCCG AGAAGATAAA GAAGAAGTAC TTGCGTACGT GGTTCGTGGT GGACTTCGTG 360

TCATCCATCC CGGTGGACTA CATCTTCCTC ATAGTGGAGA AGGGAATCGA CTCCGAGGTC 420

TACAAGACAG CGCGTGCTCT GCGCATCGTG CCGTTCACCA AGATCCTCAG TCTGCTGCGG 480

CTGCTGCGGC TATCACGGCT CATCCGATAT ATCCACCAGT GGGAAGAGAT TTTCCACATG 540

ACCTACGACC TGGCAAGTGC AGTGATGCGC ATCTGTAACC TGATCAGCAT GATGCTACTG 600

CTCTGCCACT GGGACGGTTG CCTGCAGTTC CTGGTGCCCA TGCTGCAAGA CTTCCCCAGC 660

GACTGCTGGG TGTCCATCAA CAACATGGTG AACCACTCGT GGAGCGAGCT CTACTCGTTC 720

GCGCTCTTCA AGGCCATGAG CCACATGCTG TGCATCGGCT ACGGGCGGCA GGCGCCCGAG 780

AGCATGACAG ACATCTGGCT GACCATGCTC AGCATGATCG TAGGCGCCAC CTGCTATGCC 840

ATGTTCATTG GGCACGCCAC TGCGCTCATC CAGTCCCTGG ATTCGTCACG GCGCCAATAC 900

CAGGAGAAGT ACAAGCAAGT AGAGCAATAC ATGTCCTTCC ACAAACTGCC CGCTGACTTC 960

CGCCAGAAGA TCCACGATTA CTATGAACAC CGGTACCAAG GGAAGATGTC TGATGAGGAC 1020

AGCATCCTTG GGGAACTCAA CGGGCCACTG CGTGAGGAGA TTGTGAACTT CAACTGCCGG 1080

AAGCTGGTGG CTTCCATGCC GCTGTTTGCC AATGCAGACC CCAATTTCGT CACAGCCATG 1140

CTGACAAAGC TCAAATTTGA GGTCTTCCAG CCTGGAGATT ACATCATCCG AGAGGGGACC 1200

ATCGGGAAGA AGATGTACTT CATCCAGCAT GGGGTGGTGA GCGTGCTCAC CAAGGGCAAC 1260

AAGGAGATGA AGCTGTCGGA TGGCTCCTAT TTCGGGGAGA TCTGCTTGCT CACGAGGGGC 1320

CGGCGTACGG CCAGCGTGCG AGCTGACACC TACTGTCGCC TCTACTCACT GAGTGTGGAC 1380

AATTTCAACG AGGTGCTGGA GGAATACCCC ATGATGCGGC GTGCCTTTGA GACTGTGGCT 1440

ATTGACCGGC TAGATCGCAT AGGCAAGAAG AACTCCACCT TGCTGCACAA GGTTCAGCAT 1500

GATCTCAGCT CCACGCCGCG CCTGGGACCC GCACCCACCG CCCGGACCGC CGCGCCCAGT 1560

CCGGACCGCA GGACTCAGGG AATT 1584

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 528 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Lys Phe Ser Leu Arg Met Phe Gly Ser Gln Lys Ala Val Glu Arg Glu
1 5 10 15

Gln Glu Arg Val Lys Ser Ala Gly Ala Trp Ile Ile His Pro Tyr Ser
20 25 30

Asp Phe Arg Phe Tyr Trp Asp Phe Thr Met Leu Leu Phe Met Val Gly
35 40 45

Asn Leu Ile Ile Ile Pro Val Gly Ile Thr Phe Phe Lys Asp Glu Thr
50 55 60

Thr Ala Pro Trp Ile Val Phe Asn Val Val Ser Asp Thr Phe Phe Leu
65 70 75 80

Met Asp Leu Val Leu Asn Phe Arg Thr Gly Ile Val Ile Glu Asp Asn
85 90 95

Thr Glu Ile Ile Leu Asp Pro Glu Lys Ile Lys Lys Lys Tyr Leu Arg
100 105 110

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Thr Trp Phe Val Val Asp Phe Val Ser Ser Ile Pro Val Asp Tyr Ile
        115                 120                 125

Phe Leu Ile Val Glu Lys Gly Ile Asp Ser Glu Val Tyr Lys Thr Ala
    130                 135                 140

Arg Ala Leu Arg Ile Val Pro Phe Thr Lys Ile Leu Ser Leu Leu Arg
145                 150                 155                 160

Leu Leu Arg Leu Ser Arg Leu Ile Arg Tyr Ile His Gln Trp Glu Glu
                165                 170                 175

Ile Phe His Met Thr Tyr Asp Leu Ala Ser Ala Val Met Arg Ile Cys
            180                 185                 190

Asn Leu Ile Ser Met Met Leu Leu Leu Cys His Trp Asp Gly Cys Leu
        195                 200                 205

Gln Phe Leu Val Pro Met Leu Gln Asp Phe Pro Ser Asp Cys Trp Val
    210                 215                 220

Ser Ile Asn Asn Met Val Asn His Ser Trp Ser Glu Leu Tyr Ser Phe
225                 230                 235                 240

Ala Leu Phe Lys Ala Met Ser His Met Leu Cys Ile Gly Tyr Gly Arg
                245                 250                 255

Gln Ala Pro Glu Ser Met Thr Asp Ile Trp Leu Thr Met Leu Ser Met
            260                 265                 270

Ile Val Gly Ala Thr Cys Tyr Ala Met Phe Ile Gly His Ala Thr Ala
        275                 280                 285

Leu Ile Gln Ser Leu Asp Ser Ser Arg Arg Gln Tyr Gln Glu Lys Tyr
    290                 295                 300

Lys Gln Val Glu Gln Tyr Met Ser Phe His Lys Leu Pro Ala Asp Phe
305                 310                 315                 320

Arg Gln Lys Ile His Asp Tyr Tyr Glu His Arg Tyr Gln Gly Lys Met
                325                 330                 335

Ser Asp Glu Asp Ser Ile Leu Gly Glu Leu Asn Gly Pro Leu Arg Glu
            340                 345                 350

Glu Ile Val Asn Phe Asn Cys Arg Lys Leu Val Ala Ser Met Pro Leu
        355                 360                 365

Phe Ala Asn Ala Asp Pro Asn Phe Val Thr Ala Met Leu Thr Lys Leu
    370                 375                 380

Lys Phe Glu Val Phe Gln Pro Gly Asp Tyr Ile Ile Arg Glu Gly Thr
385                 390                 395                 400

Ile Gly Lys Lys Met Tyr Phe Ile Gln His Gly Val Val Ser Val Leu
                405                 410                 415

Thr Lys Gly Asn Lys Glu Met Lys Leu Ser Asp Gly Ser Tyr Phe Gly
            420                 425                 430

Glu Ile Cys Leu Leu Thr Arg Gly Arg Arg Thr Ala Ser Val Arg Ala
        435                 440                 445
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Asp Thr Tyr Cys Arg Leu Tyr Ser Leu Ser Val Asp Asn Phe Asn Glu
450 455 460

Val Leu Glu Glu Tyr Pro Met Met Arg Arg Ala Phe Glu Thr Val Ala
465 470 475 480

Ile Asp Arg Leu Asp Arg Ile Gly Lys Lys Asn Ser Thr Leu Leu His
485 490 495

Lys Val Gln His Asp Leu Ser Ser Thr Pro Arg Leu Gly Pro Ala Pro
500 505 510

Thr Ala Arg Thr Ala Ala Pro Ser Pro Asp Arg Arg Thr Gln Gly Ile
515 520 525

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1790 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGAGGAGGC GGGCCCGGCG GGGGAGCCGC GCGGCAGCCA GGCCAGCTTC ATGCAGCGCC 60

AGTTCGGCGC GCTCCTGCAG CCGGGCGTCA ACAAGTTCTC GCTGCGGATG TTCGGCAGCC 120

AGAAGGCCGT GGAGCGCGAG CAGGAGCGCG TCAAGTCGGC GGGGGCCTGG ATCATCCACC 180

CGTACAGCGA CTTCAGGTTC TACTGGGACT TCACCATGCT GCTGTTCATG GTGGGAAACC 240

TCATCATCAT CCCAGTGGGC ATCACCTTCT TCAAGGATGA GACCACTGCC CCGTGGATCG 300

TGTTCAACGT GGTCTCGGAC ACCTTCTTCC TCATGGACCT GGTGTTGAAC TTCCGCACCG 360

GCATTGTGAT CGAGGACAAC ACGGAGATCA TCCTGGACCC CGAGAAGATC AAGAAAAGTA 420

TCTGCGCACG TGGTTCGTGG TGGTCTTCGT GTCCTCCATC CCCGTGGACT ACATCTTCCT 480

TATCGTGGAG AAGGGCATTG ACTCCGAGGT CTACAAGACG GCACGCGCCC TGCGCATCGT 540

GCGCTTCACC AAAATCCTCA GCCTCCTGCG GCTGCTGCGC CTCTCACGCC TGATCCGCTA 600

CATCCATCAG TGGGAGGAGA TCTTCCACAT GACCTATGAC CTGGCCAGCG CGGTGATGAG 660

GATCTGCAAT CTCATCAGCA TGATGCTGCT GCTCTGCCAC TGGGACGGCT GCCTGCAGTT 720

CCTGGTGCCT ATGCTGCAGG ACTTCCCGCG CAACTGCTGG GTGTCCATCA ATGGCATGGT 780

GAACCACTCG TGGAGTGAAC TGTACTCCTT CGCACTCTTC AAGGCCATGA GCCACATGCT 840

GTGCATCGGG TACGGCCGGC AGGCGCCCGA AAGCATGACG GACATCTGGC TGACCATGCT 900

CAGCATGATT GTGGGTGCCA CCTGCTACGC CATGTTCATC GGCCACGCCA CTGCCCTCAT 960

CCAGTCGCTG GACTCCTCGC GGCGCCAGTA CCAGGAGAAG TACAAGCAGG TGGAGCAGTA 1020

CATGTCCTTC CACAAGCTGC CAGCTGACTT CCGCCAGAAG ATCCACGACT ACTATGAACA 1080

CCGTTACCAG GGCAAGATGT TTGACGAGGA CAGCATCCTG GGCGAGCTCA ACGGGCCCCT 1140

GCGGGAGGAG ATCGTCAACT TCAACTGCCG GAAGCTGGTG GCCTCCATGC CGCTGTTCGC 1200

CAACGCCGAC CCCAACTTCG TCACGGCCAT GCTGACCAAG CTCAAGTTCG AGGTCTTCCA 1260

GCCGGGTGAC TACATCATCC GCGAAAGCAC CATCGGGAAG AAGATGTACT TCATCCAGCA 1320

CGGCGTGGTC AGCGTGCTCA CTAAGGGCAA CAAGGAGATG AAGCTGTCCG ATGGCTCCTA 1380

CTTCGGGGAG ATCTGCCTGC TCACCCGGGG CCGCCGCACG GCGACGTGCG GGCTGACACC 1440

TACTGCCGCC TCTATTCCCT GAGCGTGGAC AACTTCAACG AAGTGCTGGA GGAGTACCCC 1500

ATGATGCGGC GCGCTTTCGA GACGGTGGCC ATCGACCGCC TGGACCGCAT CGGCAAGAAG 1560

AATTCCATCC TCCTGCACAA GGTGCAGCAT GACCTCAACT CGGGCGTATT CAACAACCAG 1620

GAGAACGCCA TCATCCAGGA GATCGTCAAG TACGACCGCG AGATGGTGCA GCAGGCCGAG 1680

CTGGGTCAGC GCGTGGGCTT TTTCCCGCCG CCGCCGCCGC CGCCGCAGGT CACTTCGGCC 1740

ATCGCCACGC TGCAGCAGGC GGCGGCCATG AGCTTCTGCC CGCAGGTGGC 1790

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 598 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Xaa Glu Glu Ala Gly Pro Ala Gly Glu Pro Arg Gly Ser Gln Ala Ser
1 5 10 15

Phe Met Gln Arg Gln Phe Gly Ala Leu Leu Gln Pro Gly Val Asn Lys
20 25 30

Phe Ser Leu Arg Met Phe Gly Ser Gln Lys Ala Val Glu Arg Glu Gln
35 40 45

Glu Arg Val Lys Ser Ala Gly Ala Trp Ile Ile His Pro Tyr Ser Asp
50 55 60

Phe Arg Phe Tyr Trp Asp Phe Thr Met Leu Leu Phe Met Val Gly Asn
65 70 75 80

Leu Ile Ile Ile Pro Val Gly Ile Thr Phe Phe Lys Asp Glu Thr Thr
85 90 95

Ala Pro Trp Ile Val Phe Asn Val Val Ser Asp Thr Phe Phe Leu Met
100 105 110

Asp Leu Val Leu Asn Phe Arg Thr Gly Ile Val Ile Glu Asp Asn Thr
115 120 125

Glu Ile Ile Leu Asp Pro Glu Lys Ile Lys Xaa Lys Tyr Leu Arg Thr
130 135 140

Trp Phe Val Val Val Phe Val Ser Ser Ile Pro Val Asp Tyr Ile Phe
145 150 155 160

Leu Ile Val Glu Lys Gly Ile Asp Ser Glu Val Tyr Lys Thr Ala Arg
165 170 175

Ala Leu Arg Ile Val Arg Phe Thr Lys Ile Leu Ser Leu Leu Arg Leu
180 185 190

Leu Arg Leu Ser Arg Leu Ile Arg Tyr Ile His Gln Trp Glu Glu Ile
195 200 205

Phe His Met Thr Tyr Asp Leu Ala Ser Ala Val Met Arg Ile Cys Asn
210 215 220

Leu Ile Ser Met Met Leu Leu Leu Cys His Trp Asp Gly Cys Leu Gln
225 230 235 240

Phe Leu Val Pro Met Leu Gln Asp Phe Pro Arg Asn Cys Trp Val Ser
245 250 255

Ile Asn Gly Met Val Asn His Ser Trp Ser Glu Leu Tyr Ser Phe Ala
260 265 270

Leu Phe Lys Ala Met Ser His Met Leu Cys Ile Gly Tyr Gly Arg Gln
275 280 285

Ala Pro Glu Ser Met Thr Asp Ile Trp Leu Thr Met Leu Ser Met Ile
290 295 300

Val Gly Ala Thr Cys Tyr Ala Met Phe Ile Gly His Ala Thr Ala Leu
305 310 315 320

Ile Gln Ser Leu Asp Ser Ser Arg Arg Gln Tyr Gln Glu Lys Tyr Lys
325 330 335

Gln Val Glu Gln Tyr Met Ser Phe His Lys Leu Pro Ala Asp Phe Arg
340 345 350

Gln Lys Ile His Asp Tyr Tyr Glu His Arg Tyr Gln Gly Lys Met Phe
355 360 365

Asp Glu Asp Ser Ile Leu Gly Glu Leu Asn Gly Pro Leu Arg Glu Glu
370 375 380

Ile Val Asn Phe Asn Cys Arg Lys Leu Val Ala Ser Met Pro Leu Phe
385 390 395 400

Ala Asn Ala Asp Pro Asn Phe Val Thr Ala Met Leu Thr Lys Leu Lys
405 410 415

Phe Glu Val Phe Gln Pro Gly Asp Tyr Ile Ile Arg Glu Ser Thr Ile
420 425 430

Gly Lys Lys Met Tyr Phe Ile Gln His Gly Val Val Ser Val Leu Thr
435 440 445

Lys Gly Asn Lys Glu Met Lys Leu Ser Asp Gly Ser Tyr Phe Gly Glu
450 455 460

Ile Cys Leu Leu Thr Arg Gly Arg Arg Thr Ala Xaa Val Arg Ala Asp
465 470 475 480

Thr Tyr Cys Arg Leu Tyr Ser Leu Ser Val Asp Asn Phe Asn Glu Val
485 490 495

Leu Glu Glu Tyr Pro Met Met Arg Arg Ala Phe Glu Thr Val Ala Ile
500 505 510

Asp Arg Leu Asp Arg Ile Gly Lys Lys Asn Ser Ile Leu Leu His Lys
515 520 525

Val Gln His Asp Leu Asn Ser Gly Val Phe Asn Asn Gln Glu Asn Ala
530 535 540

Ile Ile Gln Glu Ile Val Lys Tyr Asp Arg Glu Met Val Gln Gln Ala
545 550 555 560

Glu Leu Gly Gln Arg Val Gly Phe Phe Pro Pro Pro Pro Pro Pro Pro
565 570 575

Gln Val Thr Ser Ala Ile Ala Thr Leu Gln Gln Ala Ala Ala Met Ser
580 585 590

Phe Cys Pro Gln Val Ala
595

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1507 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGCAACAGCC CTCGGCGGAC ACCGCTATCA AAGTGGAGGG AGGCGCGGCC GCCACACCAT 60

ATCCTCCCCG AGGCCGAGTG CGCCTGGGCC AAAGCGGCTT CATGCAGCGC CAGTTCGGTG 120

CCATGCTGCA ACCTGGGGTC AACAAATTCT CCCTAAGGAT GTTCGGCAGC CAAAAGCGGT 180

GGAGCGCGAG CAGGAGAGGG TTAATCAGCA GGGTTTTGGA TTATCCACCC CTACAGTGAC 240

TTCAGATTTT ACTGGGACCT GACATCTGTT GCTGATGGTG GGGAATCTGA TCATCATACC 300

CGTGGGCATC ACCTTCTTCA AGGATGAGAA CACCACACCC TGGATCGTCT TCAATGTGGT 360

GTCAGACACA TTCTTCCTCA TTGACTTGGT CCTCAACTTC CGCACGGGGA TCGTGGTGGA 420

GGACAACACA GAAATCATCC TTGACCCGCA GAGGATCAAG ATGAAGTACC TGAAAAGCTG 480

GTTTGTGGTA GATTTCATCT CCTCCATACC TGTCGAATAC ATTTCCTTAT AGTGGAGACT 540

CGCATTGACT CGGAGGTTTA CAAAACCGCT AGGGCTGTGC GCATTGTCCG TTTCATAAGA 600

TCCTCAGCCT CCTGCGCCTC TTGAGGCTTT CCCGCCTCAT TCGATACATT CATCAGTGGG 660

AAGAGATTTT CCACATGACC TATGACCTGG CCAGCGCCGT GGTACGCATC GTGAACCTCA 720

TTGGCATGAT GCTTCTGCTG TGTCACTGGG ATGGCTGCCT GCAGTTCCTA GTGCCCATGC 780

TGCAGGACTT CCCCCATGAC TGCTGGGTGT CCATCAATGG CATGGTGAAT AACTCCTGGG 840

GGAAGCAGTA TTCCTACGCC CTCTTCAAGG CCATGAGCCA CATGCTGTGC ATTGGGTATG 900

GACGGCAGGC ACCCGTAGGC ATGTCTGACG TCTGGCTCAC CATGCTCAGC ATGATCGTGG 960

GGGCCACCTG CTATGCCATG TTCATCGGCC ACGCCACTGC CCTCATCCAG TCGCTAGACT 1020

CCTCCCGGCG CCAGTACCAG GAGAAGTATA AACAGGTGGA GCAGTACATG TCTTTCCACA 1080

AGCTCCCGCC TGACACCCGA CAGCGCATCC ATGACTACTA TGAACACCGT TACCAAGGCA 1140

AGATGTTTGA TGAGGAAAGC ATCCTGGGTG AGTTGAGTGA GCCACTTCGA GAGGAGATCA 1200

TCAACTTTAA CTGCCGAAAG CTGGTGGCAT CCATGCCACT GTTTGCCAAC GCAGATCCCA 1260

ACTTTGTGAC ATCCATGCTG ACCAAGTTGC GTTTCGAGGT CTTCCAGCCT GGGGATTACA 1320

TCATCCGCGA AGGCACCATC GGCAAGAAGA TGTACTTTAT CCAGCACGGC GTGGTCAGCG 1380

TGCTCACTAA GGGCAACAAA GAGACCAGGC TGGCTGATGG CTCCTATTTT GGAGAGATCT 1440

GCTTGCTGAC CCGGGGTCGG CGCACAGCCA GCGTCAGAGC GGATACTTAT TCCGCCTCTA 1500

CTCACTG 1507

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 506 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Cys Xaa Gln Pro Ser Ala Asp Thr Ala Ile Lys Val Glu Gly Gly Ala
1 5 10 15

Ala Ala Xaa Xaa His Ile Leu Pro Glu Ala Xaa Val Arg Leu Gly Gln
20 25 30

Ser Gly Phe Met Gln Arg Gln Phe Gly Ala Met Leu Gln Pro Gly Val
35 40 45

Asn Lys Phe Ser Leu Arg Met Phe Gly Ser Xaa Lys Ala Val Glu Arg
50 55 60

Glu Gln Glu Arg Val Xaa Ser Ala Gly Phe Trp Ile Ile His Pro Tyr
65 70 75 80

Ser Asp Phe Arg Phe Tyr Trp Asp Leu Thr Xaa Leu Leu Leu Met Val
85 90 95

Gly Asn Leu Ile Ile Ile Pro Val Gly Ile Thr Phe Phe Lys Asp Glu
100 105 110

Asn Thr Thr Pro Trp Ile Val Phe Asn Val Val Ser Asp Thr Phe Phe
115 120 125

Leu Ile Asp Leu Val Leu Asn Phe Arg Thr Gly Ile Val Val Glu Asp
130 135 140

Asn Thr Glu Ile Ile Leu Asp Pro Gln Arg Ile Lys Met Lys Tyr Leu
145 150 155 160

Lys Ser Trp Phe Val Val Asp Phe Ile Ser Ser Ile Pro Val Glu Tyr
165 170 175

Xaa Phe Leu Ile Val Glu Thr Arg Ile Asp Ser Glu Val Tyr Lys Thr
180 185 190

Ala Arg Ala Val Arg Ile Val Arg Phe Xaa Lys Ile Leu Ser Leu Leu
195 200 205

Arg Leu Leu Arg Leu Ser Arg Leu Ile Arg Tyr Ile His Gln Trp Glu
210 215 220

Glu Ile Phe His Met Thr Tyr Asp Leu Ala Ser Ala Val Val Arg Ile
225 230 235 240

Val Asn Leu Ile Gly Met Met Leu Leu Leu Cys His Trp Asp Gly Cys
245 250 255

Leu Gln Phe Leu Val Pro Met Leu Gln Asp Phe Pro His Asp Cys Trp
260 265 270

Val Ser Ile Asn Gly Met Val Asn Asn Ser Trp Gly Lys Gln Tyr Ser
275 280 285

Tyr Ala Leu Phe Lys Ala Met Ser His Met Leu Cys Ile Gly Tyr Gly
290 295 300

Arg Gln Ala Pro Val Gly Met Ser Asp Val Trp Leu Thr Met Leu Ser
305 310 315 320

Met Ile Val Gly Ala Thr Cys Tyr Ala Met Phe Ile Gly His Ala Thr
325 330 335

Ala Leu Ile Gln Ser Leu Asp Ser Ser Arg Arg Gln Tyr Gln Glu Lys
340 345 350

Tyr Lys Gln Val Glu Gln Tyr Met Ser Phe His Lys Leu Pro Pro Asp
355 360 365

Thr Arg Gln Arg Ile His Asp Tyr Tyr Glu His Arg Tyr Gln Gly Lys
370 375 380

Met Phe Asp Glu Glu Ser Ile Leu Gly Glu Leu Ser Glu Pro Leu Arg
385 390 395 400

Glu Glu Ile Ile Asn Phe Asn Cys Arg Lys Leu Val Ala Ser Met Pro
405 410 415

Leu Phe Ala Asn Ala Asp Pro Asn Phe Val Thr Ser Met Leu Thr Lys
420 425 430

Leu Arg Phe Glu Val Phe Gln Pro Gly Asp Tyr Ile Ile Arg Glu Gly
435 440 445

Thr Ile Gly Lys Lys Met Tyr Phe Ile Gln His Gly Val Val Ser Val
450 455 460

Leu Thr Lys Gly Asn Lys Glu Thr Arg Leu Ala Asp Gly Ser Tyr Phe
465 470 475 480

Gly Glu Ile Cys Leu Leu Thr Arg Gly Arg Arg Thr Ala Ser Val Arg
485 490 495

Ala Asp Thr Tyr Xaa Arg Leu Tyr Ser Leu
500 505

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1060 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTTTTTTTTT TTTTTTTTTT TTGGGTTTTA AAATTTATTT TATTTTTAAA AGCGTCTCCG 60

GAATCTAGTG CATGGCCAGG CTACAAGCTA CTGGGCCAGC AACTCTGTAG GATTATTAAT 120

GACAAAAATG CAAGGACCCC ATAGTTGATG GAAACCCAGG GATGAAGCAG GGCTGTCCCA 180

CAGACTTAGG CTTTGTGGAG CTGTCTGAAA ACCCAGGCTG TGGCTTTGGA AGAAGTGCAG 240

ACAACCACTG CCCAGAGTGA CTTAAGGTTC ATACAACCAT CCAGCCACCT AAGCACCCCT 300

ACCTTCAAGC ATCTTGCCAG TCCCACTTTG TGTCTGTTTA GCCTGCTTTT CTCCTCCCAA 360

GTTAGGAGTC GGGTACACCC TGGGACGGAG CAATAAGACT GGGGTTGGAG TTAATGTGTA 420

AAATAACTGA AAAAAACATC TGGGGCTGGC AAACCTGTTT GTCTGGAAAA CAGCCTTCCA 480

GATGTGCAGG TATGGAAACA GACAGTGCTT AGAGCAGTAA GGGACCTTAT ACCAGCTAAT 540

CGTTCATTCT CCCAAGTATA AGGAGGAATC TGGGGGTGCT GGGTTAGCTG CTGCAGGCCT 600

AATTGGGGGG TGGAATGGGA GCTCTGAGCT CTTCCCCGCT TTCGCAGAGA TCTGCCTGCT 660

GATTCGAGGT CGGAGAACAG CCAGTGTAAG GGCTGACACC TATTGTCGCC TTACTCGCTC 720

AGCGTGGACC ACTTCAATGC GGTGCTTGAG GAGTTCCCAA TGATGCGCAG GGCTTTTGAG 780

ACGGTGGCCA TGGACCGGCT TCGGCGCATC GGTGAGGCCT GTTTACTCTG TCTGCTCTGG 840

GTCCTGGCTG GGCCTCATCT CATGAGCCTA GCCCTGGTGC TTTGACACCA CATCCCAGCC 900

CACCCAGTTC CAGTCCATGC CTCCAGCAGG CTGTTAGCAC TGTTGCTCAC TAGACTTAGC 960

CCTAGCGAGA AATTGCCGTG GAGTGTCTCC CCAAACCCTC ATTCCCCGTG TCTTCTGGGT 1020

ACCAGTTCTT AACCTCACAA TTTTTTATTG ATACCTCGTG 1060

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 79 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Glu Ile Cys Leu Leu Ile Arg Gly Arg Arg Thr Ala Ser Val Arg Ala
1 5 10 15

Asp Thr Tyr Cys Arg Leu Tyr Ser Leu Ser Val Asp His Phe Asn Ala
20 25 30

Val Leu Glu Glu Phe Pro Met Met Arg Arg Ala Phe Glu Thr Val Ala
35 40 45

Met Asp Arg Leu Arg Arg Ile Gly Glu Ala Cys Leu Leu Cys Leu Leu
50 55 60

Trp Val Leu Ala Gly Pro His Leu Met Ser Leu Ala Leu Val Leu
65 70 75

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGAGGCATAG TAGCCACCAG TTTCC 25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCGCTCGAGG CCTTGGTATC GGTGCTCATA G 31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAAGCGGATG TTAACGATAC CAGCC 25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GACAAGCCGA CAACCTTGAT TGGAG 25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAGCAAGTTC AGCCTGGTTA AGTCC 25

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTGGCTTATG AGTATTTCTT CCAGGG 26

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGGGAAGAGA TATTCCACAT GACC 24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TACGACCTGG CAAGTGCAGT GATGCGC 27

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTTCACAAT CTCCTCACGC AGTGGCCC 28

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTGGTGGATA TATCGGATGA GCCG 24

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CAGTGGGAAG AGATTTTCCA CATGACC 27

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GATCATGCTG AACCTTGTGC AGCAAG 26

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CACCKCRTTG AAGTGGTCCA CGCT 24

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATGTTCGGSA GCCAGAAGGC GGTGGAG 27

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CAGCTCGAAC ACTGGCAGTA CGAC 24

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AACTTCAACT GCCGGAAGCT GGTG 24

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GAAAAAGCCC ACGCGCTGAC CCAG 24

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CACCAGCTTC CGGCAGTTGA AGTTG 25

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCGAATTCAA ACCCAACTCC GCGTCCAA 28

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCTGAATTCA CTGTACGGAT GGAT 24

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GTCGTACTGC CAGTGTTCGA GCTG 24

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GGTCAGGTTG GTGTTGTGAA ACGC 24

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GAGCAGGAGC GCGTCAAGTC GGCG 24

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GAAGATGTAG TCCACGGGGA TGGA 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/27630

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00, 5/00, 1/20, 5/06; C07K 16/00, C12Q 1/68, 19/42
US CL :435/320.1, 325, 252.3, 69.1, 6, 86, 336; 530/388.22
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 325, 252.3, 69.1, 6, 86, 336; 530/388.22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, BIOTECHDS, SCISEARCH, LIFESCI, HCAPLUS, WPIDS, NTIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Genbank-est109, Accession No. N72770, HILLIER et al., 'The Washu-Merck EST project', 23 February 1996, see nucleic acids 1-1401 of the EST for 100% identity to a portion of BCNG protein.	1-10,13, 23
X	WANG et al. The seizure locus encodes the Drosophila homolog of the HERG Potassium Channel. J. Neuroscience. 01 February 1997, Vol. 17, No. 3, pages 882-890, see the abstract and the properties of their Potassium Channel, and compare to BCNG protein.	14-21, 60-62

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 APRIL 1999	Date of mailing of the international search report 05 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARYAM MONSHIPOURI Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/27630

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WILLIAMSON et al. Isolation of the structural gene for alcohol dehydrogenase by genetic complementation in yeast. Nature. 10 January 1980, pages 214-216, compare their nucleic acids 608-632 to SEQ ID NO: 16.	24 and 32
X	Database Genbank 110, Accession No. M24665, J02862, VANDESLICE et al. 'Dog mastocytoma protease mRNA', 15 September 1989, see their nucleic acids 14-38 and compare to SEQ ID NO:17 of this application.	24 and 32
X	BIEL et al. Molecular cloning and expression of a modulatory subunit of the cyclic Nucleotide-gated cation Channel. J. Biol. Chem., 15 March 1996, Vol.15, No.11, pages 6349-6355, see the entire article specially figure 4-5.	36, 37-40, 42-44, 50-51
X	PEDARZANI et al. Protein kinase A-independent modulation of ion channels in the brain by cyclic AMP., Proc. natl. Acad. Sci. U.S.A., 10 December 1995, Vol.92, pages 11716-11720, see the whole document and figs 1-2.	35
X,P	SANTORO et al. Identification of a gene encoding a hyperpolarization-activated pacemaker Channel of brain. Cell. 29 May 1998, Vol. 93, pages 717-729, see the entire article.	1-1-40, 42-44, 50-51, 60-62
X	SANTORO et al., Interactive cloning with the SH3 domain of N-src identifies a new brain specific ion channel, with homology to Eag and cyclic nucleotide-gated channels. Proc. Natl. Acad. Sci. USA. 10 December 1997, Vol. 94, pages 14185-14820, see the whole article.	1-40, 42-44, 50-51, 60-62

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/27630

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 41, 45-49 and 52-59
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 41 (and its dependent claims 52-59) as well as claim 45 (and its dependent claims 46-49) refer to a method of identifying an "unknown compound". A method of identifying an unknown compound does not make sense and therefore cannot be searched.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.